The role of NF-κB regulation of eosinophil apoptosis and survival; Implications for the pathogenesis of Hodgkin's disease

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Abstract

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In inflammatory diseases such as asthma inappropriately recruited or activated eosinophilic granulocytes may cause damage to the tissue in the respiratory tract by release of their toxic contents. This can be resolved by efficient removal of activated eosinophils from inflammatory sites. Ligation of specific cell surface receptors and the consequent triggering of diverse signal transduction pathways control eosinophil functional responsiveness and physiological programmed cell death or apoptosis. During apoptosis eosinophils undergo a series of characteristic changes (e.g. shrinkage, chromatin condensation, expression of cell surface phosphatidylserine) to shutdown their secretory capacity of toxic cell contents and marks them for 'silent' removal from inflamed sites by macrophages. However, the precise role of apoptosis in eosinophilic inflammatory disease and other diseases where eosinophils are observed in abundance (e.g. Hodgkin's disease) has not been fully elucidated.

Apoptosis is regulated by extracellular stimuli, such as TNFα, FasL, and their cell surface receptors by activating various death pathways such as the caspase pathway. However, the pro-inflammatory cytokine TNFα can also trigger the NF-κB pathway for transcriptional activity which is responsible for synthesis of survival and pro-inflammatory proteins. There is a delicate balance between these dual pathways, pro-survival and pro-apoptotic, generated by TNFα. Inhibition of TNFα -mediated NF-κB activation ultimately un-masks the caspase-dependent pro-apoptotic properties of TNFα. In particular, the role of NF-κB activation in the regulation of eosinophil survival and the potential contribution of apoptosis to eosinophils presence found in Hodgkin's disease was investigated.

Activation of NF-κB is mediated by signal-induced phosphorylation via the IKK complex by TNFα and degradation of its inhibitor, IκBα in the cytoplasm. Degradation of IκBα in the cytoplasm by TNFα and translocation of NF-κB into the nucleus were determined by immunofluorescence and western blotting analysis. Various pharmacological reagents (e.g., the fungal metabolite gliotoxin and the proteasome inhibitor Mg132) and the HIV-1-TAT transduction peptide linked with IκBα were used to stabilise and over-express IκBα in the cytoplasm resulting in the prevention of translocation of NF-κB into nucleus. The 11 amino acid TAT peptide linked with super-suppressive form of IκBα (IκBα32,36) was for the first time produced and transduced into eosinophils, HeLa and A549 cell lines, and resulted in an inhibition of NF-κB. Inhibition of TNFα mediated IκBα degradation and NF-κB activation by gliotoxin, Mg132 and TAT- IκBα32,36 induced significant eosinophil apoptosis and prevented NF-κB regulated IL-8 production. It was notable however that the effect of TAT-IκBα32,36

was donor dependent, in that some populations of eosinophils failed to undergo enhanced apoptosis, suggesting the involvement of other pathways distinct from NF-κB.

Hodgkin's disease (or Hodgkin's lymphoma) is characterised by a minority (about 1% of tumour mass) of neoplasic cells, the so called Hodgkin-Reed-Sternberg (HRS) cells, and where the 'tumour' mass is comprised of predominantly recruited eosinophils. However, the mechanisms responsible for this eosinophil accumulation are currently unknown. *In vitro*, eosinophils cultured with supernatant derived from HRS cells caused a profound survival of eosinophil (e.g., supernatant treated eosinophils survived up to 6 times longer than non-treated eosinophils). This effect was blocked by various NF-κB inhibitors which caused eosinophil apoptosis, indicating the potential of NF-κB as a target for anti-tumour therapy in Hodgkin's disease.

In summary, these data strongly suggest the important role of NF-κB in controlling eosinophil responsiveness and apoptosis which may provide alternative therapeutic agents for the treatment of eosinophilic diseases, including asthma, eczema, rhinitis and Hodgkin's disease where eosinophils are present in abundance.

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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Publications

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Contents

Abbreviations

AA; arachidonic acid
AHR; airways hyperreactivity
AIDS; acquired immunodeficiency syndrome
AIF; apoptosis inducing factor
AMP; ampicillin
AP-1; Activator Protein 1
ARDS; acute respiratory distress syndrome
ARM; arginine rich motif
BAF; bronchial lavage fluid
BAL; broncho alveolar lavage
BH domain; BCL-2 homology domain
bp; base pairs
BSA; bovine serum albumin
cDNA; complementary DNA
CED; cell death genes
DC; dendritic cells
DMEM; Dulbecco's modified Eagle's medium
DMSO; dimethyl suphoxide
DTT; dithiothrietol
E.*coli*; *Eschericia coli*
EBV; Estein-Barr virus
ECP; eosinophil cationic protein,
EDN; eosinophil-delived neurotoxin
EDTA; Ethylenediaminetetracetic acid
ELISA; enzyme-linked immunosorbent assay
EPO; eosinophil peroxidase
ERK; extracellular regulated kinase
FADD; Fas-associated death domain protein
FasL; Fas ligand
FCS; foetal calf serum
FITC; fluorescent isothiocyanate
fMLP; N- formyl- methionyl-leucyl-phenylalanine, bacterial products;
GM-CSF; granulocyte-macrophage colony-stimulating factor
GST; glutathione S-transferase
HES; hypereosinophil syndrome
HIV; human immunodeficiency virus
HRS cell; Hodgkin-Reed-Sternberg cell
HS; heparan surface
HUVEC; human umbilical vein endothelial cells
IAP; inhibitor of apoptosis
ICAM; intracellular adhesion molecule
ICE; interleukin-1β-converting enzyme
Ig; Immunoglobulin
IKK; IκB kinase
IL; interleukin
IκB; inhibitor of κB
JNK; c-Jun N-terminal protein kinase
KDa; kilo Dalton
LB; luria-bartani broth
LD; lymphocyte-depleted (HD)
LP; lymphocyte predominant (HD)
LPS; lipopolysaccharide
LRP; lipoprotein receptor-related protein
LT; lymphotoxin
LTC4; leukotriene C4 synthase
LTR; long terminal repeat
MAP kinase; mitogen activated protein kinase
MBD; macrophage-derived chemokine
MBP; majour basic protein
MC; mixed cellularity (HD)
MIP; macrophage inflammatory protein
MW; molecular weight
NADPH oxidase; nicotinamide adenine dinucleotide phosphate oxidase
NF-κB; nuclear factor-κB
NLS; nucleus localisation signals
NP-40; Nonidet P-40
NS; nodular sclerosis (HD)
PAF; platelet-activating factor
PAGE; polyacrylamide gel electrophoresis
PBS; phosphate buffered saline
PCD; programmed cell death
PCR; polymerase chain reaction
PI; propidium iodide
PI-3 kinase; phophatidylinositol 3-kinase
PMN; polymorphonuclear neutrophils
PSGL-1; P-selectin glycoprotein ligand-1

PTD; protein transduction domain
PTP; permiability transition pore
PVDF; polyvinylidene difluoride
ROS; reactive oxygen species
S-ECP; serum level of eosinophil cationic protein
SHPTP-2; Src homology 2 phosphatase 2 tyrosine phosphatase
SUMO; small ubiquitin-like modifier
TAT-32,36IκBα; TAT-IκBα mutated serine 32 and 36 to Alanine
TAT-WTIκBα; TAT-IκBα wild type
TNFα; tumor necrosis factor α
TNFR; TNF receptor
TRADD; TNFR-associated death domain protein
TRAF; TNF-associated factor
UV; ultra-violet
VCAM; vascular cell adhesion molecule
WB; Western blotting
WT; wild type
XIAP; X-chromosome-linked inhibitor of apoptosis
zVAD-fmk; z-Val-Ala-DL-Asp-fluoremethylketone

Chapter layout and aims are;

Chapter 1; General Introduction. This introduction summarises the knowledge to date on the function of eosinophils in inflammatory diseases. Then the mechanism regulating apoptosis and the NF-κB pathway is summarised.

Chapter 3; The methodology to investigate the function of NF-κB in granulocytes. Methodology of studying granulocyte apoptosis was investigated. This is continuous work from our previous publication Ward *et al.*, 1999 (see appendix for the paper).

Chapter 4; The inhibition of NF-κB un-mask the ability of TNFα to induce apoptosis in eosinophils. This study was investigated by the usage of NF-κB inhibitors, gliotoxin and Mg132. This chapter is based on my previous publication, Fujihara *et al.*, 2002, (see appendix for the paper.)

Chapter 5; Use of TAT-IκBα for the inhibition of NF-κB in HeLa, A549 and eosinophils, and its effect on eosinophil apoptosis. This study was investigated by the usage of the transduction peptide HIV-1-TAT linked with IκBα to inhibit specifically NF-κB in HeLa, A549 cells and eosinophils. The effect of NF-κB inhibition and apoptosis in eosinophils are investigated.

Chapter 6; Hodgkin's disease and eosinophilia. The observation of enhanced survival of eosinophils by Hodgkin-Reed-Sternberg cell lines and possible mechanisms regulatory tumour formation.

Chapter 1
General Introduction

1. General Introduction

1.1. Eosinophil

1.1.1. Eosinophil

Eosinophils are thought to be key cells involved in host defence and are particularly effective against helminth parasite worm infections (Butterworth *et al.*, 1975, Gleich and Adolphoson, 1986). On the other hand, excessive eosinophil recruitment or activation at inflammatory sites likely causes surrounding tissue damage by liberation of their granules toxic contents and by release of pro-inflammatory cytokines and chemokines. Therefore, effective removal of eosinophils from inflammatory sites may be a key factor for successful resolution of the inflammatory process.

Eosinophils are terminally differentiated leukocytes which are derived from bone marrow, where approximately the mean turn over of eosinophils is approximately $2.2x10^8$ cells/kg/day (Parwaresch *et al.*, 1979). In healthy donors, 2-5% among the peripheral leukocytes are eosinophils. Once in the circulation, the half life of eosinophils is approximately 18 hours. However, the half-life of eosinophils is prolonged during specific circumstances, including inflammation. A typical view of eosinophils under light microscopy is shown in figure 4-2-5-A and B (chapter 4). Eosinophils are approximately 8μm in diameter, containing typically a bi-lobed nucleus, which can be stained with blue dye, haematoxylin. In the cytoplasm one-fifth is occupied with ovoid granules, stained orange by eosin, which appear according to the maturation status of the cells.

Eosinophil and parasite

The role of cell-mediated response against helminths is mostly dependent on the activation of eosinophils which release toxic granules to damage the outer surface of the helminths. Helminths are large multicellular organisms. Several *Schistosoma* species are responsible for the chronic, debilitating and sometimes fatal disease schistosomiasis. Eosinophils are thought to be the first defence against helminthic infection. Once helminthic infection occurs in a human subject, the total IgE levels can exceed those seen in asthmatic and atopic dermatitis patients, sometimes up to 40 x 10^9/ l (Yazdanbakhsh, 1996). As shown in figure 1-1-1, activation of Th2 pathway releases Th2 cytokines, IL-4, IL-13 and IL-5, which results in activation and degranulation of eosinophils. Eosinophil degranulation is activated by the binding of IgA and IgG to the eosinophil. The complement cascade also results in the degranulation of eosinophils. C3a is an eosinophil chemoattractant and binding of C3a promotes release of EPO, EDN and ECP which may be enhanced by IL-3 and IL-5 (Takafuji *et al.,* 1994, and 1996, Bach *et al.,* 1990). Once eosinophils are activated their toxic contents MBP, ECP and EDN are released which damage the outer surface of parasites. For example, MBP increases membrane permeability (Young *et al.*, 1986) and perturbation of the lipid bilayer of the target cells (Wasmoen *et al.*, 1988).

Eosinophil granules

Granules are categorised as secondary granules, small granules, primary granules and lipid bodies. (See table 1-1-1 for summarised contents of granules in eosinophil.) These granules contain not only many highly toxic proteins with powerful enzymatic activity but also certain cytokines which can promote the inflammatory response. For example, secondary granules contain the cytokines; TNFα, GM-CSF, IL-2, IL-4, IL-6

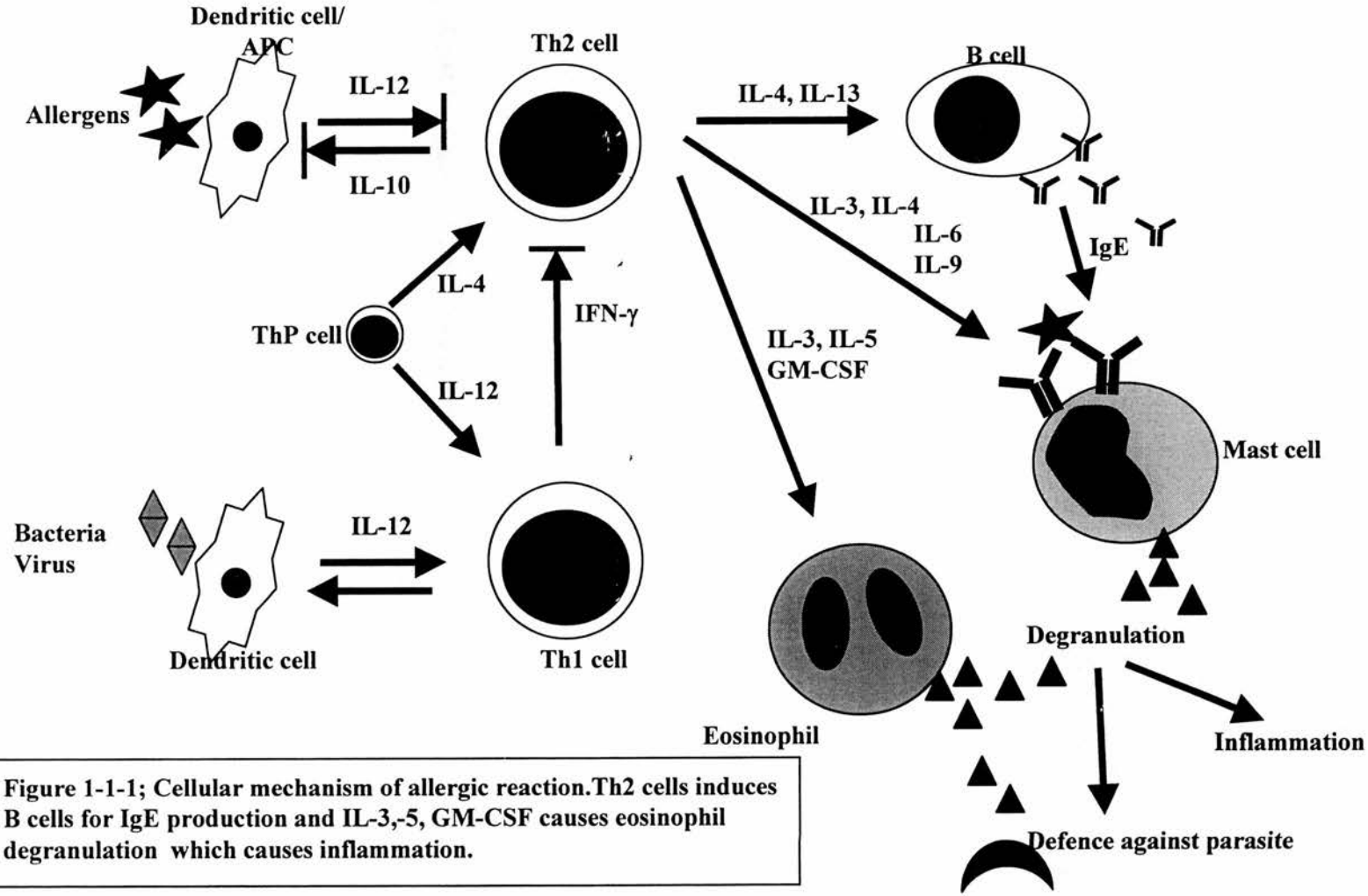


Figure 1-1-1; Cellular mechanism of allergic reaction.Th2 cells induces B cells for IgE production and IL-3,-5, GM-CSF causes eosinophil degranulation which causes inflammation.

and proteases; major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO). These products can promote the progress of inflammation in diseases such as asthma. Also lipid bodies contain arachidonic acid (AA) and leukotriene C4 synthase (LTC4), which also have pro-inflammatory properties.

granules	**Secondary granules**	**Small granules**	**Primary granules**	**Lipid bodies**
contents	MBP ECP EPO EDN TNFα GM-CSF IL-2 IL-4 IL-6	Elastase gelatinase (MMP9)	CLC-P	eicosanoid AA LTC4 synthase

Table 1-1-1; Summary of contents of eosinophil granules (modified from Giembycz and Lindsay, 1999)

Eosinophilic toxic proteins are important for defence against parasites, however, if toxic contents are released in the host tissue, when eosinophils are activated, this will contribute to the progress of inflammation. For instance, MBP (Kroegel *et al.*, 1987), ECP and EPO (Frigas *et al.*, 1981) are known to be toxic to mammalian cells and may produce pathological changes through alteration of lipid membranes *in vitro*.

In particular, in asthma, primed eosinophils release ECP, EDN, MBP and oxygen free radicals may be one of the direct causes of airway remodelling. Increased air wall thickness and decreased airway caliber were observed when airway sections were incubated with EPO from PAF-activated eosinophils (Rabe *et al.*, 1994). MBP is

thought that to bind to anionic domains of target parasites resulting in the pertubation of the lipid bilayer. *In vitro*, MBP increases $[Ca^{2+}]i$ in bovine tracheal smooth muscle (TSM) (Wylam *et al*, 1998) and guinia pig TSM contractions (Streck *et al.*, 1996). In primate both MBP and EPO induce AHR (Gundel *et al*, 1991). In asthmatic subjects, MBP (Frigas *et al.*, 1981) and elastase (Lungarella *et al.*, 1992) were found in significantly increased amounts in the airway, which indicates the airway tissue damage done by those proteases.

Another function of eosinophils is the production of some pro-inflammatory proteins including IL-1 (Weller *et al.*, 1993), IL-4 (Moqbel *et al.*, 1995, Nonaka *et al.*, 1995), IL-6 (Hamid *et al.*, 1992), IL-8 (Braun *et al.*, 1993, Kita *et al.*, 1995) TNFα (Costa *et al.*, 1993), GM-CSF (Weller *et al.*, 1992), IL-5 (Broide *et al.*, 1992, Dubucquoi *et al.*, 1994). The synthesis of the proteins is dependent on the activation of intracellular signalling cascades including activation of key transcription factors. Some of these cytokines trigger the activation of inflammatory cells including eosinophils thereby contributing to an amplification of the inflammatory response and disease progression.

1.1.2. Intracellular signalling pathways

Eosinophils are one of the cell types where tyrosine cascades is crucial for cell activation and survival. IL-5, IL-3, GM-CSF (Simon *et al,* 1997), which all share receptors partially, platelet-activating factor (PAF) (Miike *et al.*, 2000), interferon-γ (IFN-γ) (Ochiai *et al.*, 1999) activates Lyn, JAK2 kinases, PI-3, Syk and Src homology 2 phosphatase 2 tyrosine phosphatase (SHPTP-2). Blocking phosphorylation of JAK2 by specific inhibitors induces eosinophil apoptosis, indicating tyrosine cascades that are crucial for eosinophil survival (Yousefi *et al.*, 1996, Pazdrak *et al.*, 1997). IL-5

induced activation of ERK1 and ERK2 resulting in leukotriene C4 synthesis (Bates *et al.*, 2000).

The three MAP kinase families, extracellular regulated kinases (ERKs), the c-jun N-terminal kinases/ stress-activated protein kinases, and p38 MAP kinases have common functions including cellular activation in various cell types, including eosinophils. The ERKs are activated mainly by mitogens such as growth factors and G-protein coupled receptor agonists whereas c-jun N-terminal kinase and p38 are activated by various cellular stresses. Activated p38 phosphorylates and stimulates down stream kinase, mitogen-activated protein kinase-activated protein kinases (MAPKAPKs) (Rouse *et al.*, 1994). In summary, IL-5-induced activation of those cascades and pathways exert an anti-apoptosis effect in eosinophils (Owen *et al.*, 1989, Rothernberg *et al.*, 1989, Pazdrak *et al.*, 1995, Yousefi *et al.*, 1996).

Distinct from tyrosine cascades, as previously shown from my study (Fujihara *et al.*, 2002) (see chapter 4 and appendix for the paper) and others (Yamashita *et al.*, 1999) NF-κB regulates eosinophil activation (e.g. production of IL-8) and survival. Applying NF-κB inhibitors factors such as the proteasome inhibitor Mg132 and the fungal metabolite gliotoxin significantly induced eosinophil apoptosis and suppressed the production of IL-8, indicating that NF-κB is an important transcription factor that regulates cellular activation and apoptosis. (See NF-κB section in 1.5 and the introduction part in chapter 4 for more details, especially the mechanism of Mg132 and gliotoxin.)

1.2. Neutrophils

Neutrophils are the first cell type to be recruited at sites of bacterial or fungal infection and are thought to contribute to the progression of damage to healthy tissue. For instance, in adult respiratory destress syndrome (ARDS), neutrophils may damage tissue through H2O2 generation (McGuire *et al.*, 1982). Recent evidence suggests that not only eosinophils but also neutrophils are involved in asthma pathogenesis. For instance, in non-allergic asthma substances, such as nitrogen dioxide and ozone (Coffey *et al.*, 1996, Fahy *et al.*, 1995) and respiratory tract infection (Lamblin *et al.*, 1998) induces an influx of neutrophils into the airways. Study of status asthmaticus by Tonnel *et al.*, (2001) showed dramatic increases in neutrophils and neutrophil elastase in bronchoalveolar lavage (BAL). Also, the level of EPO and gelatinase (MMP9) were increased in those samples, indicating that eosinophil degranulation also plays a role in the neutrophil influx seen in status asthmaticus patients.

1.3. Inflammatory diseases

1.3.1. Inflammation and allergy

Inflammatory diseases such as asthma, eczema and other allergic diseases are increasing in both developed and developing countries. In Western countries 155 million individuals (up to 10 to 20 % of children's population) are affected by asthma and eczema (reviewed by Sampson *et al.*, 2000).

Inflammation is a local response to injury, involving vasodilatation and increased permeability of capillaries in damaged areas largely due to release of substances such as histamine and serotonin from mast cells. Vasodilation also helps recruitment of inflammatory cells, such as macrophages, T cells, B cells and particularly

granulocytes, which have the potential to cause damage and destroy surrounding tissue. Neutrophils are the first cells to migrate at inflammatory sites to defend against invading micro-organisms and are often seen at acute inflammatory sites, where eosinophils are more commonly seen at sites of parasitic infection and are associated with chronic allergic diseases.

Allergic reaction (hypersensitivity) and inflammation

Hypersensitivity is classified into four types, but it is important to realise that a great deal more complexity exists due to the vast array of secondary effects which cross the boundaries of the classification scheme.

Type I hypersensitivity is IgE-mediated and its initiation time is between 2 - 30 minutes. The Ag induces cross-linkage of IgE bound to mast cells and basophils with release of vasoactive mediators. This typically manifests as systemic anaphylaxis, or localised anaphylaxis (e.g., hay fever, asthma, hives, food allergies, eczema). When a host body is exposed to an allergen (such as grass, ragweed, penicillin, local anaesthetics, nuts, seafood, bee venom, wasp venom, house dust mite, mold spores, animal hair and dander) B cells are activated to form IgE-secreting plasma cells. The secreted IgE molecule binds IgE-specific Fc receptors on mast cells and blood basophils. Upon a second exposure to the allergen, the bound IgE is cross-linked, triggering the release of pharmacologically active mediators, such as histamine, serotonin, platelet-activating factor (PAF), LT, prostaglandins, TNFα, IL-1, IL-5, GM-CSF, from mast cells and basophils. These mediators cause smooth-muscle contraction, increased vascular permeability, and vasodilation which results in the allergic reaction such as asthma.

Type I hypersensitivity is commonly identified and assessed by skin testing. If a person is allergic to the allergen, local mast cells will degranulate. The release of histamine and other mediators produces a wheal and flare within 30 min. Similarly, radioallergosorbent test (RAST) or radioimmunosorbent test (RIST) are commonly used for type I hypersensitivity test. Several drugs block the release of allergic mediators by interfering at various biochemical steps which are involved in the activation and degranulation of mast cells. For example, disodium chromoglycate prevents Ca^{2+} influx into mast cells. Theophylline, which is commonly administered orally or through inhalers to asthmatics, blocks phosphodiesterase, which catalyses the conversion of cAMP to 5'-AMP. The resulting prolonged increase in cAMP levels blocks degranulation. A number of drugs stimulate the beta-adrenergic system by stimulating beta-receptors. Epinephrine is commonly administered during anaphylactic shock. It acts by binding to beta receptors on bronchial smooth muscles and mast cells, elevating the cAMP levels within these cells. The increased levels of cAMP leads to relaxation of the bronchial muscle and decreased mast-cell degranulation. Corticosteroids and other anti-inflammatory drugs also have been used to reduce type I reactions.

Type II hypersensitivity is an antibody-mediated cytotoxic reaction, initiation time is between 5 and 8 hours after exposure to the allergen. The mechanism involves the production of antibody which directs against cell-surface destruction via complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC). Typical manifestations are blood-transfusion reactions, erythroblastosis fatalist, autoimmune haemolytic anaemia. Type III hypersensitivity reaction is immune complement-

mediated hypersensitivity where has an initiation time is between 2- 8 hours. Antibody and antigen complex deposited in various tissue induce complement activation and an inflammatory response. Typical manifestations are localized arthus reacations, serum sickness, rheumatoid arthritis, systemic lupus erythematousus. Type IV hypersensitivity in sensitised CD4+ T_{DTH} cells which release cytokines that activate macrophages or Tc cells which mediate direct cellular damage by lytic enzyme released by activated macrophages. Its initiation time is 24- 72 hours as 'delayed' reaction. Typical manifestations are contact dermatitis, tubercular lesions, and graft rejection.

T cells and B cells in inflammation

The developmental process that results in generation of antibody-secreting B cells can be divided into two phases; an antigen-independent phase, which occurs in the bone marrow, and an antigen-dependent phase, which occurs in the periphery. Following release of mature B cells from the bone marrow, the subsequent steps in B-cell development and differentiation occur in the periphery and require antigen. Activated B cells can undergo class switching resulting in expression of isotypes other than IgM and IgD on the membrane. As a result, memory B cells often express membrane IgG, IgA, or IgE.

IL- 4 and 13 are the type 2 cytokines which have a broad spectrum of reactivity, especially mediating IgE synthesis (See figure 1-1-1). Both IL-4 and IL-13 shear the alpha chain of the IL-4 receptor (IL-4Rα). Th2 cells, mast cells, basophils and eosinophils are capable for producing IL-4, however, the relevant source of IL-4 for IgE synthesis in allergic reaction is unclear.

IL-4 exerts different effects on B cells at different stages in the cell cycle. On resting B cells - IL-4 acts as an activating factor - inducing the resting cells to enlarge in size and increase their expression of Class II MHC.Then it acts as a growth factor, driving the B cells to proliferate. Finally, IL-4 acts as a differentiation factor by regulating the class switch to IgG1 and then to IgE "switch-inducing factor" Ligand initiated binding on IL-4Rα activates the signalling cascade signal transducer and transcription –6 (STAT-6) for transcription and synthesis of mRNA. However, other receptors such as CD40 and CD80/ 86 cause a switch to IgE synthesis. The interaction of CD40-CD154 (T cells) activates NF-κB to enhance further IgE production by Stat-6 pathway (Warren *et al.*, 1999)

A shift in the class switching in B cells occurs by recombination between the VHDHJH region and downstream constant regions specific for each class in heavy chain DNA. The constant region for the chain (C) lies upstream of the constant regions for all the other heavy chains. Thus an IgM-producing B cell (at antigen-independent phase) can switch to the production of immunoglobulin classes under certain stimuli to activate B cells. However, a cell producing IgG cannot switch to IgM production because its DNA has previously undergone a recombination event which eliminates the C region.

1.3.2. Asthma and inflammatory cells

Asthma is an inflammatory disease of the airways of the lungs. The airways narrow due to smooth musculature contraction and airways hyperreactivity (AHR) with mucous hypersecretion. Accumulation of eosinophils in the lungs is a common feature

of asthma, although neutrophilia has been reported in fatal acute asthma (Fahy *et al.*, 1995).

Asthma and eosinophils

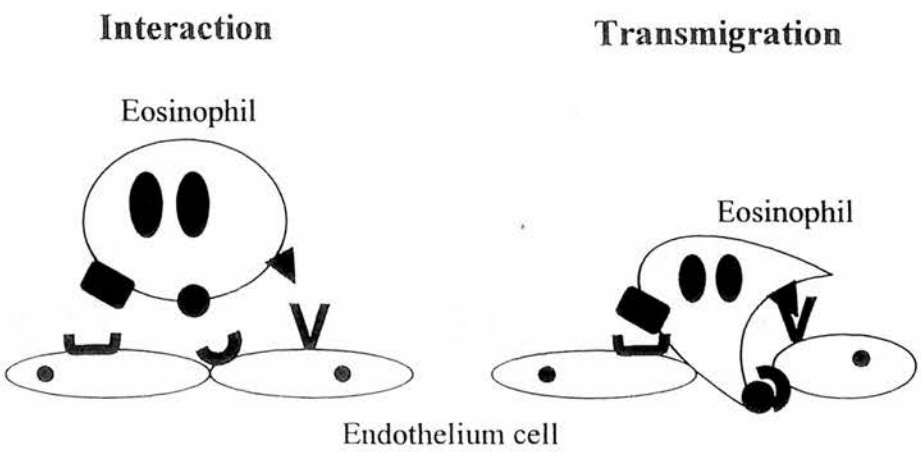
Tissue eosinophilia is a characteristic of asthma and there appears to be a positive correlation between the AHR and number of eosinophils found in the BAL fluid and peripheral blood of patients with asthma (Bousquet *et al.*, 1990, Bradley *et al.*, 1991). In the airway, activated eosinophils release toxic products such as MBP, ECP, EDN and oxygen free radicals, which damage the surrounding tissue resulting in the contraction of bronchial smooth muscle (Rabe *et al.*, 1994), increased vascular permeability (Collins *et al,* 1993) and induction of AHR (Leff, 1994).

The mechanisms regulating asthma are complex and involve various types of cells such as neutrophils, T cells, B cells, mast cells, macrophages, epithelial cells, myofibroblasts, smooth muscles, and especially eosinophils (Bousquet *et al.*, 1990, Djukanovic *et al.*, 1990). These cells are recruited by released cytokines and chemokines from many cells at the inflammatory sites. For instance, in BAL fluid increased amounts of IL-4 (Shi *et al.*, 1990), IL-5 (Webb *et al.*, 2000), IL-13, TNFα (Luttman *et al.*, 1999), MCP-4, IL-16 (Taha *et al.*, 2001), MCP-1, MIP-α, RANTES (Alam *et al.*, 1996) have been reported. Furthermore, recruited inflammatory cells interact with other cells such as endothelial cells via ICAM-1 and VCAM-1 (Granger and Kubes, 1994, Bochner and Schleimer, 1994) resulting in the activation of the cells.

Administration of IL-13 in the airways of mice increases AHR, BAL eosinophil influx, serum IgE and mucus secretion. These actions were reversed by blocking the IL-13 receptors (Uills-Karp *et al.*, 1998, Crünig *et al.*, 1998). In addition, IL-13 was demonstrated to mediate PSGL-1/ P-selectin dependent adhesion of eosinophils to HUVECS (Woltmann *et al.*, 2000). Furthermore, both IL-4 and IL-13, which share a common IL-4-receptor α subunit, stimulate epithelial cell release of eotaxin, a known strong eosinophil chemoattractant (Li *et al.*, 1999) and can activate B cells to release IgE which is captured by Fc receptors (FcεRI and FcεRII) on eosinophils.

The recruitment of eosinophils occurs at the capillary level where flow is slow enough to allow cells to roll through endothelial cells. The mechanism of infiltration and accumulation of inflammatory cells at inflammation may be due to release of various chemoattractants from surrounding cells in the tissue, such as fibroblasts, and inflammatory cells themselves. Furthermore, following the recruitment of those inflammatory cells, there may be some substances released resulting in high cellular activation and enhanced survival of inflammatory cells. As a consequence activated inflammatory cells are able to successfully escape from the normal induction of apoptosis which results in clearance of dying cells by macrophage phagocytes. Table 6-1-7-A summarise the eosinophilic chemoattractant cytokines and chemokines are listed according to their molecular weight.

Among various cytokines and chemokines as shown in table 6-1-7-B, some chemokines, such as eotaxin, IL-8, TRAC, RANTES, and cytokines, such as TNFα, TGFβ, IL-2, IL-3, IL-5, GM-CSF and IL-13 modulate various aspects of eosinophil function. Among them are eosinophil chemoattractants such as IL-2, RANTES, IL-8



eosinophil surface molecule	eosinophil surface molecule (CD)	receptors
PECAM-1	CD31	PECAM-1
α2β1	CD49b/ CD29	Laminin ?
αLβ2	CD11a/CD18	ICAM-1 ICAM-2 ICAM-3
αMβ2	CD11b/ CD18	ICAM-1 ICAM-3 Fibrinogen iC3b
αXβ2	CD11c/CD18	Fibinogen iC3b
αdβ2	ad/CD18	ICAM-3
α4β1	CD49d/CD18	VCAM-1 Fibronectin
α4β7	CD49d/CD103	VCAM-1 MAdCAM-1 Fibronectin
L-selectin	CD62L	Glycam-1 CD34 MAdCAM-1
PSGL-1		E-Selectin P-Selectin
α5β1	CD49e/CD29	Fibronectin
α6β1	CD41/CD29	Laminin
LAF-1		ICAM-1
VLA-4		VCAM-1
CR-3		ICAM-1

Table 1-3-2; The summary of eosinophil surface molecules and their binding receptors (modified from Gimbycz and Lindsay, 1999).

and eotaxin. Eosinophil activators are most of the chemokines and cytokines such as IL-3, IL-5, GM-CSF, IL-13, TNFα, TGFβ. In addition, cytokines which are known to influence eosinophil survival include IL-3, IL-5, GM-CSF, IL-13, TNFα. The selective and existing chemoattractants near inflammatory sites cause the eosinophils to be primed and to interact with endothelial cells through particular interaction with surface molecules for cell rolling (see table 1-3-2).

The expression of adhesion molecules on the surface of eosinophils is essential for migration. The summary of eosinophil adhesion molecules is shown in table 1-3-2. Selective migration occurs in three steps; rolling of eosinophils along the surface of the vessels, firm adhesion of eosinophils with endothelial cells and transmigration of eosinophils into tissues. Various evidence suggests that for eosinophil rolling, expression of L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) are essential for interaction to E- and P-selectin on the surface of endothelial cells. Especially, P-selectin is more potent for eosinophil rolling (Symon *et al.*, 1996) binding to PSGL-1 (Patel and McEver, 1997). *In vivo*, P-selectin deficient mice reduced the infiltration of eosinophils in the lungs and BAL after allergen-challenge (Broide *et al.*, 1998b). Several studies have demonstrated that the expression of very late activation antigen 4 (VLA-4), CR3 and lymphocyte function-associated antigen 1 (LFA-1) on eosinophils, VCAM-1, ICAM-1 and E-selectin on endothelial cells are particularly important for adhesion of eosinophils to endothelial cells (Walsh *et al.*, 1990, Kyan Aung *et al.*, 1991a,b, Weller *et al.*, 1991, Nakajima *et al.*, 1994). In asthmatic individuals ICAM-1 and HLA-DR are significantly expressed in eosinophils and epithelial cells respectively in BAL (Hansel *et al.*, 1991, Mengelers *et al.*, 1994) and VLA-4, CR3, LFA-1 in eosinophil rich mucosa and ICAM-1, VCAM-1 and E-selectin on the luminal

membrane of endothelial cells (Ohkawara *et al.*, 1995). IL-5, which is highly expressed in asthmatic airways (Leff *et al.*, 1991) downregulates L-selectin but upregulates beta2-integrins on the surface of eosinophils. On the surface of endothelial cells, the ligands for eosinophil surface integrins are the immnoglobulin supergenes (e.g., ICAM-1 ,VCAM-1). IL-4 (Schleimer *et al.*, 1992) and IL-13 (Sironi *et al.*, 1994, Bochner *et al.*, 1995) upregulate VCAM-1 expression on endothelial cells. The expression of adhesion molecules may cause some functional activation in eosinophils. VLA-4-mediated adhesion interacts with VCAM-1 and fibronectin. Upon VLA-4 stimulation, eosinophils augment PAF- and A23187-induced LTC_4 release (Anwar *et al*, 1994, Munoz *et al*, 1996), fMLP-induced ECP release (Neeley *et al.*, 1994), IL-3, GM-CSF- induced eosinophil survival (Anwar *et al.*, 1993) and NADPH oxidase release by the interaction of VLA-4/VCAM-1 (Nagata *et al.*, 1995a) and VLA-4/fibronectin (Neeley *et al.*, 1994). In monkies, anti-ICAM-1 antibody reduces the number of eosinophils in BAL, and AHR after bronchial airway challenge by methacholine (measuring PC_{100}) (Wegner *et al.*, 1990). This data indicates that the infiltration of primed eosinophils is an important part of AHR in asthma.

Anti-IL-5 antibody and asthma

IL-5 has a variety of influences on eosinophils. IL-5 promotes the terminal differentiation of eosinophils in bone marrow, prolongs the viability of mature eosinophils and primed eosinophils. Several studies demonstrate that there is a correlation between between IL-5 expression and eosinophil influx, eosinophil counts in the peripheral blood and severity of asthma (Corrigan and Kay, 1990, reviewed by Hamelmann and Gelfand, 2001).

Leckie *et al.*, (2000) has demonstrated that a single intravenous infusion of monoclonal anti-IL-5 antibody did not protect against the allergen-induced late asthmatic response and did not have any effects on baseline or post-allergen airway hyperresponsiveness (AHR), despite a dramatic decrease in peripheral blood and sputum eosinophil count. However there is slight protection seen in histamine PC_{20} in pre-allergen challenge at day 8. This data indicates a disassociation between eosinophil number in peripheral blood and sputum, and AHR. Eosinophil numbers are one of the indications of the progress of inflammation and asthma (Green *et al.*, 2002). However, in Leckie's study peripheral blood and sputum eosinophil number were examined but not eosinophil number in BAL or bronchial tissue, nor the level of activation of eosinophils, which may be the more direct indication of residual eosinophil numbers in the airways. In other studies, infusions of humanised anti-IL-5 monoclonal antibody (mepolizumab) to mild atopic asthmatics decreased airway eosinophil numbers (Food-Page *et al.,* 2003), the marker of airway remodelling (Food-Page *et al.*, 2003), hyper-eosinophilic syndromes in tissue (Garrett *et al.,* 2004), bone marrow eosinophil maturation (Menzies-Gow *et al.*, 2003) and peripheral eosinophil number (Food-Page *et al.*, 2003, Garrett *et al.*, 2004, Buttner *et al.*, 2003). However, in these studies there is no direct evidence of improvement of symptoms of asthma. In subjects with severe persistent asthma treated with oral or high dose of inhaled steroids, infusion of humanised anti-human IL-5 antibody (SCH55700) has improved FEV1 and circulation of eosinophil counts (Kips *et al.*, 2003).

In vivo, there are contrasting results on the function of eosinophils and AHR. Direct evidence of a link between local IL-5 and eosinophilia in the airways has been demonstrated in mice (Lee *et al.*, 1997). Hamelmann *et al.*, (1997, 2000), Eum *et al.*,

(1995) have demonstrated a correlation between the influx of eosinophils and AHR after aerosolised MCH challenges. In IL-4 deficienct mice, adeno-IL-5 transfection before allergen challenges result in a high influx of eosinophils in BAL and an increase in AHR. These results indicate the role of IL-5 as eosinophil chemoattractant to BAL and an inducer of AHR. The contrasting results on the relation between IL-5 and eosinophils may be due to the experimental protocols, antibody amount, different species and so on.

On the other hand several studies have indicated the disassociation between eosinophil numbers and AHR, e.g., in guinea-pig (Mauser *et al.*, 1993) and mice (Mathur *et al.*, 1999).

The inflammatory response is characterised by the appearance of not only eosinophils but also CD4+ Th2 cells, mast cells and eosinophils in bronchial biopsies and in BAL fluid of asthmatic patients (Robinson *et al.*, 1992, Robinson *et al.*, 1993, Azzawi *et al.*, 1992). Cortiocosteroids (Gauvreau *et al.*, 1996), cyclosporin A (Sihra *et al.*, 1997), systemic anti-IgE (Fahy *et al.*, 1997) and leukotriene antagonists (Taylor *et al.*, 1991) are able to suppress late asthmatic reactions which are generated by different types of cells. The activity of low numbers of residual eosinophils in the airway could be the target for eosinophil-directed treatment, a separate process to suppression of the late asthmatic reaction. However, the activation state of eosinophils should be equally considered, which can be measured by the level of eosinophilic proteins (e.g., ECP, MBP, IL-8) and transcription activation factors as shown in this thesis. In Leckie's study, despite reduction of eosinophil numbers from peripheral blood and sputum, the activation state of eosinophils in BAL or sputum is unstated. One could hypothesise

that even low numbers of eosinophils, if highly activated, may be enough to cause damage to the airway and further progression of airway remodelling. Therefore, the number of residual eosinophils and other inflammatory cells in the airways is the one of the indications but the activation state has to be examined.

The difference between the *in vivo* data in different species and Leckie's study may be a result of different sensitisation and challenge protocols or interpretations of altered AHR. For example, human asthma is more chronic and includes the potential for airway remodelling. *In vivo* studies are more controllable than the human trials. For example, intravenous antibody quantity in human may not be high enough to obtain the similar results from *in vivo* studies.

1.3.3. Leukotriene and asthma

Leukotriene (LT) is a potent biological lipid mediator derived from arachidonic acid (See figure 1-3-3). The cleavage of arachidonic acid by phospholipase A2 can be metabolised to eicosanoids and 5-hydroperoxy-eicosatetraenoic acid (5-HETEP) to LTA_4 by 5-lipoxynase. LTC_4 synthetase catalyses the conjugation of glutathione with LTA_4. LTC_4 is the major product of the 5-lipoxynase (5-LO) in human. *In vitro* eosinophils stimulated combination with Ca^{2+} ionophore A23187 and eosinophil priming factors (e.g., IL-3, IL-5, GM-CSF, TNFα, PAF) produce substantial amounts of LTC_4 (reviewed by Giembycz and Lindsay 1999).

5-LO is located in the cytosol at rest, but after cell adherance or stimulation with Ca^{2+} ionophore A23187, 5-LO translocated to the nuclear envelope in neutrophils (Brock *et al.*, 1997) in leukocytes (Rourzer and Kargman, 1988) and mast cells (Malaviya e al.,

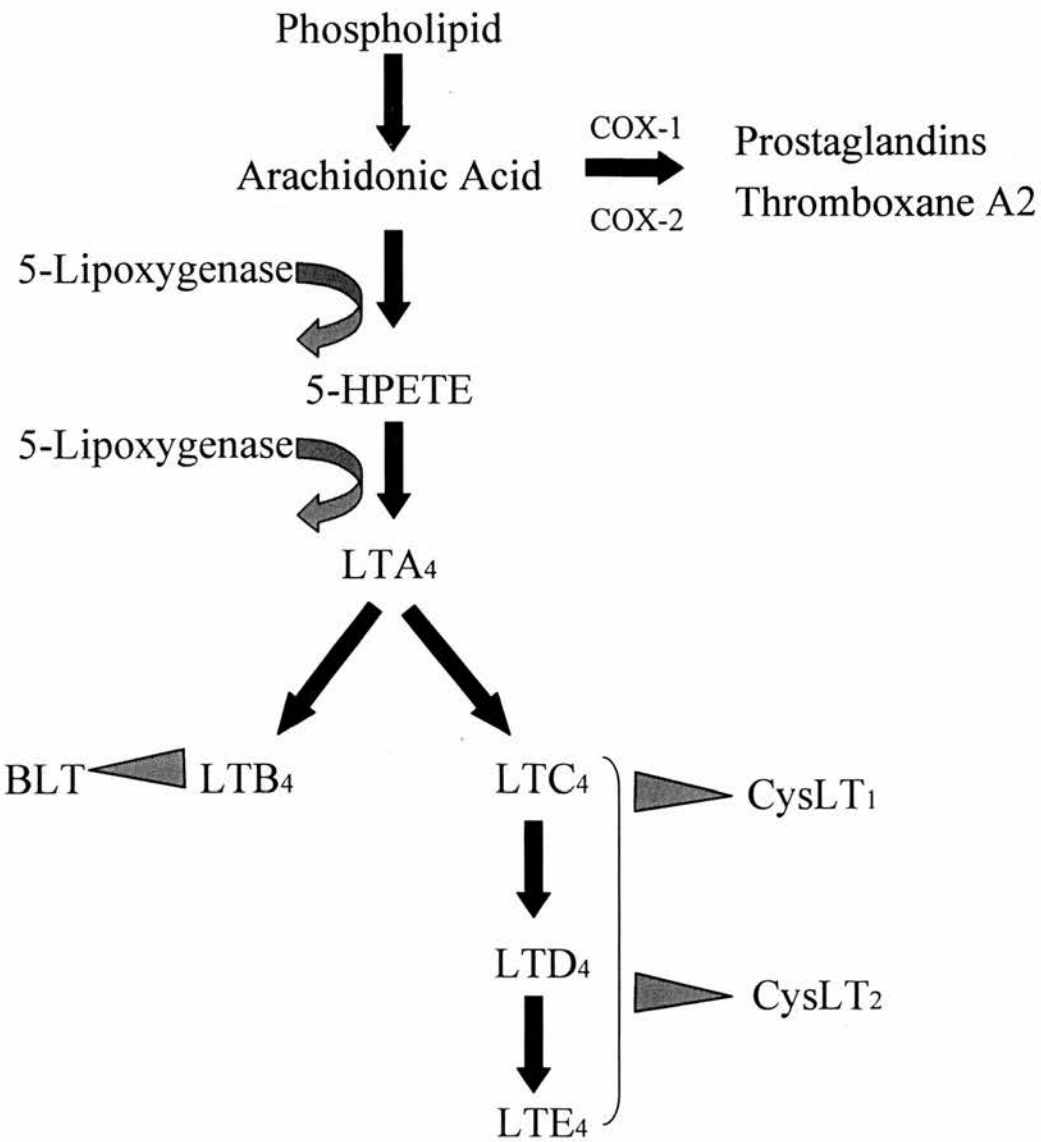


Figure 1-3-3; The pathway of leukotriene production.

1993). On the other hand, in non-stimulated rat alveolar macrophages and basophilic leukaemic cells (Coffícy *et al.*, 1992, Brock *et al.*, 1994), 5-LO is localised to the nuclear envelope, where it may be associated with the SHS3 domain of cytoskeletal proteins (Lepley and Fitzpatrick, 1994). These observations may suggest involvement of 5-LO for transcriptional activities.

In vitro, LT exert its effect to induce IL-4 from eosinophils (Banderia-Melo *et al*, 2002), induction of replication smooth muscle (Palmberg *et al.*, 1991, Rajah *et al.*, 1996) and no induction of eosinophil apoptosis (Murray *et al.*, 2003) (see Table 1-3-3-A). On the other hand, various stimuli induce LT production from eosinophils (Triggiani *et al.*, 2003, Munoz *et al.*, 2000, Banderia-Melo *et al.*, 2002, Cromwell *et al.*, 1988, Shaw *et al.*, 1984) and lung fibrobrast (Chibana *et al.*, 2003) (see Table 1-3-3-B). LT receptor antagonist (LTRA) demonstrate some roles in inhibition of primed cellular activation in eosinophils (Braccioni *et al.*, 2002, Virchow *et al.*, 2001, Suzuki *et al.*, 2003, Nagata *et al.*, 2002, Thomet *et al.*, 2001), in peripheral blood mononucleated cells (PBMNC) (Ichiyama *et al.*, 2003) and neutrophils (Koyama *et al.*, 2000, Sato *et al.*, 1999) (see Table 1-3-3-C).

Eosinophils and mast cells produce substantial amounts of LTC_4 from endogenous arachidonic acid in the airways (Shaw *et al.*, 1984). Markedly in aspirin intolerant patients, approximately 70% of all LTC_4 synthetase-positive cells in biopsy samples from airway tissue were EG2+ stained eosinophils (Cowburn 1998). In asthma, LT is mainly released from primed eosinophils, mast cells and basophils and is a potent mediator for bronchocontraction and inflammation in the airways and increased permeability of the endothelial membrane leading to edema, enhanced secretion of

authors	experiment	stimuli	cell type	effect
Bandeira-Melo C 2002	in vitro	LT	eosinophils	I. IL-4
Palmberg L 1991	in vitro	LT	smooth muscle	I. DNA replication
Rajah R 1996	in vitro	LTD_4	smooth muscle	I. MMP-1 (I. IGF activities and proliferation)
Murray J, 2003	in vitro	LTB_4 LTD_4	eosinophil neutrophil	I. $[Ca^{2+}]i$

Table 1-3-3-A. The summary of recent publications of the effect of LT on various cell types in vitro. I; increase, D; decrease

authors	experiment	stimuli	cell type	effect
Triggiani M 2003	in vitro	sPLAs	eosinophils	I. IL-6, IL-8, CD44, 69, granule enzyme NA. IL-5, LTC_4, PAF, superO anion
Munoz NM 2000	in vitro	s/n of CD-3 stimulated Th2 cells	eosinophils	I. LTC_4
Chibana K 2003	in vitro	IL-13	HFL-1 (lung fibrobrast)	I. LT1R, LTC_4, eotaxin
Bandeira-Melo C 2002	in vitro	IL-16	eosinophils	I. LTC4, lipid body, RANTES, eotaxin, IL-4
Cromwell O 1988	in vitro	grass pollen specific IgG	eosinophils	I. LTC_4
Shaw RJ 1984	in vitro		eosinophils neutrophils	I. LTC_4 from eo, LTB_4 from neu.

Table 1-3-3-B. The summary of recent publications of the effect of various stimuli on various cell types to induce the production of LT in vitro. I; increase, D; decrease, NA; not affected

authors	experiment	stimuli	cell type	effect
Braccioni F 2002	in vitro	montelukast	eo progenitor cells	D. LTD4- (IL-5, GM-CSF-) induced eo/bas colony formation from bone marrow and PB
Virchow JC Jr 2001	in vitro	montelukast	eosinophils	D. migration (IL-13, GM-CSF induced, too)
Suzuki M 2003	in vitro	pranlukast	eosinophils	D. PAF- or LTD_4-induced granule protein, LTD_4-induced superO anion
Nagata M 2002	in vitro	pranlukast	eosinophils	D. LTD_4 induced adhesion (via CD11b, CD18, ICAM, VCAM, fibronectin?)
Ichiyama T 2003	in vitro	pranlukast	U937 Jurkat PBMNC	D. IL-6 D. NF-κB
Thomet OAR 2001	in vitro	pestasin isopetasin neopetasin	eosinophils	D. GM-CSF primed LT, ECP
Koyama S 2000	in vitro	LTB_4RA	neutrophils	D. LPS-induced chemoattractant
Sato E 1999	in vitro	LTB_4RA	neutrophils macrophage	D. bleomycin-induced chemoattractant

Table 1-3-3-C. The summary of recent publications of the effect of various LT receptor antagonist (LTRA) on various cell types in vitro. I; increase, D; decrease, PB; peripheral blood

authors	experiment	stimuli	cell type	effect
Currie GP 2003	clinical	montelukast fluticasone		D. eo in PB
Diamant Z 1999	clinical	montelukast		NC. eo in sputum 24h after challenge I. PEF
Minoguchi K 2002	clinical	montelukast		D. eo in sputum I. PEF
Pizzichini E 1999	clinical	montelukast		D. eo in sputum, PB D. asthma symptoms score D. usage of β2A
Tohda Y 1999	clinical	pranlukast	PBMNC asthmatic	D, IL-4 D, IL-5 D, GM-CSF

Table 1-3-3-D. The summary of recent publications of the effect of various LT receptor antagonist (LTRA) in clinical trial I; increase, D; decrease, β2A; beta-2 agonist, PEF; peak expiratory flow.

authors	experiment	stimuli	cell type	effect
Wu AY 2003	in vivo	montelukast	lungs, BAL, blood	D. total cell no, eo, neu, lymph D. IL-4, -5, -13, eotaxin in lungs D, IL-4, -5, eotaxin in BAL D. IL-5, IgE serum no challenge studies!
Finsenes F 2002	in vivo	montelukast	BAL, lungs	D. total cell no, eo, lymph D. degree of inflam D. ET-1, IFNg (?) NC. TNF, IL-8
Finsnes F 2002	in vivo	montelukast	BAL	D, lymphocyte D, eosinophils D, ET-1 D, IFNg
Ihaku D 1999	in vivo	montelukast	BAL lung tissue	D. eo, neu, IL-5mRNA, CD3+/IL5mRNA, MBP+/IL5mRNA
Chiang N 1999	in vivo	LTB_4A		BLTR requirement for PMN chemoattractant
Kawada N 2001	in vivo	LTD_4	5-LO KO	I. eo
Hendrson 2002	in vivo	montelukast	BAL	D. eo influx

Table 1-3-3-E. The summary of recent publications of the effect of various LT receptor antagonist (LTRA) on various cell types in vivo. I; increase, D; decrease, NO; n.ot conclusive, KO; knock out.

mucus (Marom *et al.*, 1986) and increase in bronchial hyerresponsiveness (Adlroth *et al.*, 1986, O'Hickey SP *et al.*, 1991), increasing prolifereation of airway smooth muscle (Cohen P *et al.*, 1995, Panettieri RA *et al.*, 1998) and airway epithelial cells (Leikauf GD *et al.*, 1990) and induce collagenase expression in human lung fibroblasts (Medina LJ *et al.*, 1994).

LT receptor antagonist (LTRA) is selectively modify the leukotriene pathway, have exerted their effects as anti-asthma drugs in mild- moderate asthmatics improving PC20, PEF reducing eosinophils numbers in BAL induced sputum and peripheral blood (reviewed by Lipworth, 1999, reviewed by Holgate *et al.*, 1996, Currie *et al.*, 2003, Diamantz *et al.*, 1999, Minocuchi *et al.*, 2002, Pizzichini *et al.*, 1999, Tohda *et al.*, 1999, Hood *et al.*, 1999, Leff *et al.*, 1996, Wenzel *et al.*, 1990) (see Table 1-3-3-D). *In vivo*, administration of montelukast protects against asthma symptoms in ovalbumin (OVA) challenged mice significantly by reducing the airway eosinophil infiltration and the degree of inflammation (Henderson *et al.*, 2002) and other anti-inflammatory effects (Wu *et al.*, 2003, Finsenes *et al.*, 2002, Ihaku *et al.*, 1999, Chiang et a., 1999) (see table 1-3-3-E). On the other hand, OVA-sensitised 5-LO knockout mice have attenuated methacholine responsiveness, airway eosinophilia and IgE response after the challenge (Irvin *et al.*, 1997, Kawada *et al.*, 2001). These data suggest the potent role of LT in pathogenesis of asthma.

1.3.4. The role of eosinophils in airway causing smooth muscle contractions.

Eosinophils contain cationic proteins (e.g., eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil-derived neurotoxin (EDN)), eicosanoids, leukotrienes, pro-inflammatory cytokines (e.g., TNFα, IL-4, IL-8) and chemokines in their granules. If those products are released on inappropriate time, it causes host tissue damage. In

case of asthma, primed eosinophils release ECP, EDN, MBP and oxygen free radicals may be one of the direct causes of airway remodelling. Increased air wall thickness and decreased airway caliber were observed when airway sections were incubated with EPO from PAF-activated eosinophils (Rabe *et al.*, 1994). MBP is thought to bind to anionic domains of target parasite membrane resulting in the pertubation of the lipid bilayer. *In vitro*, MBP increases $[Ca^{2+}]i$ in bovine tracheal smooth muscle (TSM) (Wylam *et al*, 1998) and guinea pig TSM contractions (Streck *et al.*, 1996). In primates both MBP and EPO induce AHR (Gundel *et al*, 1991).

Eosinophils also release cytokines and chemokines such as TNFα, IL-8 and IL-4, which causes the influx of pro-inflammatory cells. This can contribute to the further progression of asthma, which is associated with airway remodelling and smooth muscle contraction.

1.4. Apoptosis

1.4.1. Morphology of apoptosis

Apoptosis or programmed cell death is essential for normal development and homeostasis to maintain physiological processes. Apoptosis is very different from necrosis, which induces swelling and rupturing of the plasma membrane, leading to the release of intracellular contents and causing damage to surrounding tissue and amplification of inflammation. Characteristic features of apoptosis include the shrinkage of the cells, exposure of phosphatidylserine on the outer plasma membrane, mitochondrial breakdown, bubble-like blebs on the cell surface and chromatin condensation (Wyllie, 1980). Apoptosis results in disassembly of the cell into membrane-enclosed vesicles called apoptotic bodies so that all the organelles are kept in the cells.

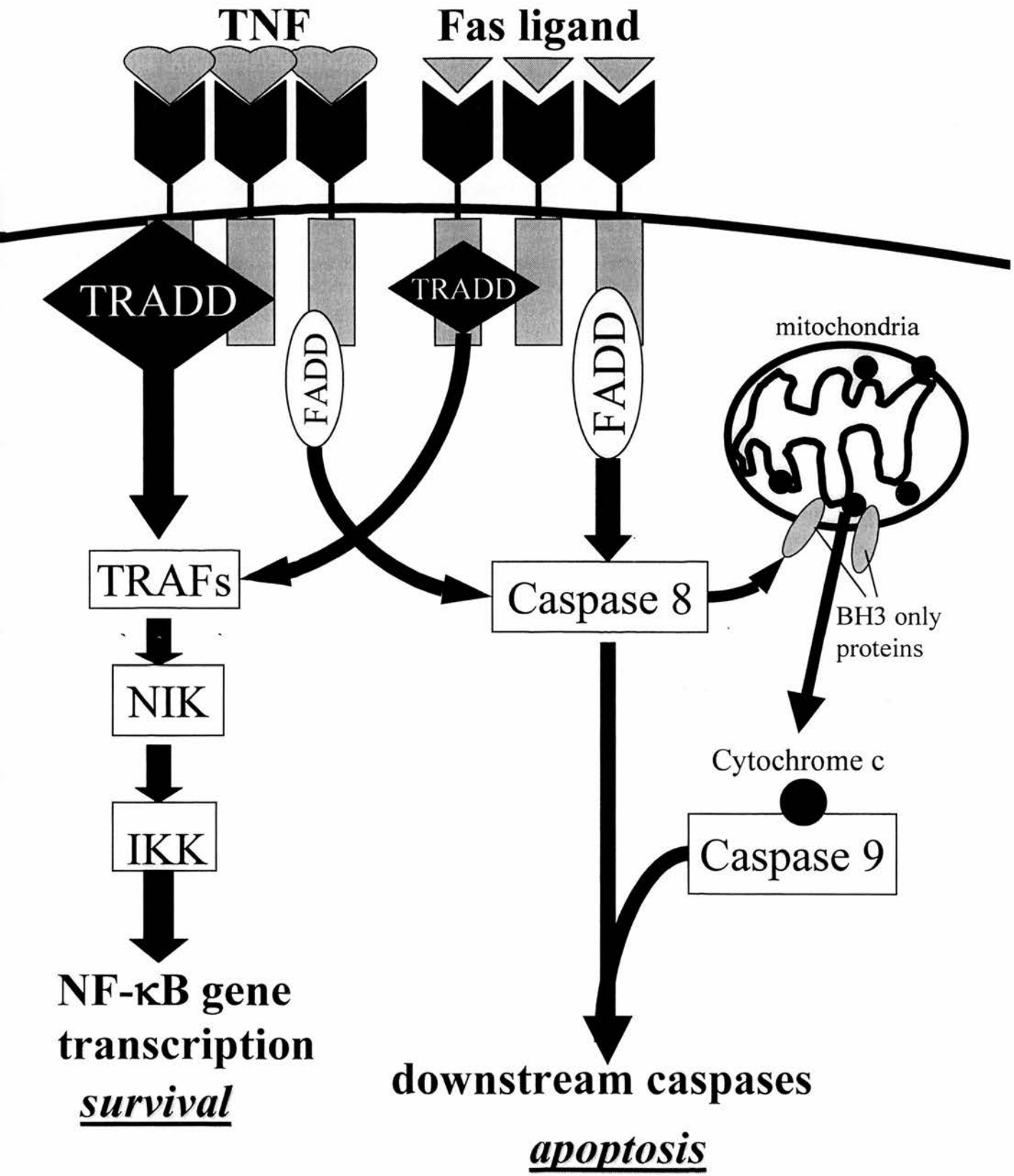


Figure 1-4-2 ; Apoptosis and the NF-κB pathway triggered by TNF superfamily receptors

1.4.2. Extracellular stimuli for the apoptosis pathway

Various extracellular stimuli from UV light to cytokines can induce cell apoptosis. TNFα and Fas ligand (Fas L) are pro-apoptotic agents which bind to the TNF superfamily of receptors; TNF receptor (TNFR) 1, TNFR2 and FAS. The TNF superfamily share a death domain (DD), which initiates caspase pathways leading to apoptosis (Itoh and Nagata, 1993, Tartaglia *et al.*, 1993, Boldin *et al.*, 1995). (See figure 1-4-2 for a summarised diagram.) The interaction of the intracellular complex leads to two major pathways, death or survival pathways. The interaction between TRAF1, TRADD and FADD results in the activation of caspase-8, which is the initiator of the downstream caspase pathway (Hsu *et al.*, 1995, Chinnaiyan *et al.*, 1995). On the other hand, TRADD also activates TRAF2 and RIP to trigger the IKK pathway leading to NF-κB and JNK activation (Natoli *et al.*, 1997). (See the following section for NF-κB activation pathway.) Therefore, the TNF superfamily can decide the fate of the cells by triggering death or survival pathways.

1.4.3. Caspase-death pathway

Caspases (cysteinyl aspartate-specific proteinases) were first implicated in apoptosis by genetic analysis in the nematode *C. elegans* (Ellis and Horvitz, 1991) and later shown to be a common feature in mammalian cells controlling the fate of the cells (Thornberry *et al.*, 1992). Caspases are expressed ubiquitously as latent pro-enzyme forms which are activated by cleavage at aspartic acid residues. This process initiates the cleavage of a cascade of caspases resulting in apoptosis.

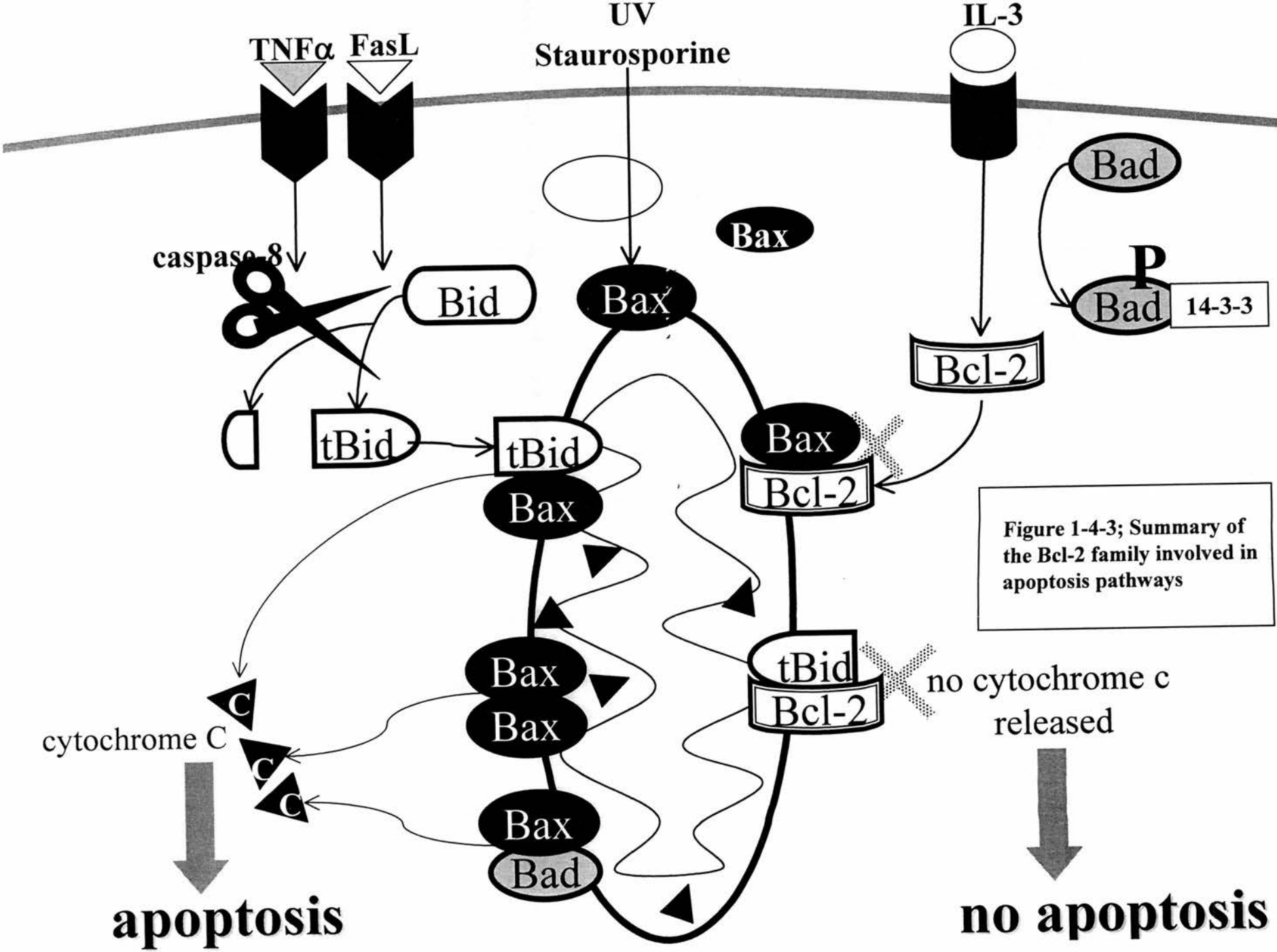


Figure 1-4-3; Summary of the Bcl-2 family involved in apoptosis pathways

The first group of the caspase cascade to be activated is caspase-8, -9, -10 after stimulation by FasL or TNFα through the TRADD and FADD complex. Caspase-8 is the initiator caspase for this cascade and not only activates other capspases, such as caspase -3, -6, -7, but also cleaves Bid to tBid which activates Bax on surface of mitochondria. (See figure 1-4-3 for the summarised diagram.) Accumulation of other pro-apoptotic Bcl-2 family members (Goping *et al.*, 1998, Antonsson *et al.*, 2001, Gross *et al.*, 1999) on the surface of mitochondria causes opening of pores in the outer and inner membrane of mitochondria to release cytochrome c which activates caspase-9, which initiates the activation of Apaf-1. Caspase-9 and Apaf-1 activation are the last steps of the caspase pathway for the biochemical execution of the cells (Slee *et al.*, 1999).

1.5. Granulocyte apoptosis

Granulocytes undergo constitutive apoptosis. However, at chronic inflammatory sites, induction of apoptosis in granulocytes may be beneficial for the successful resolution of inflammation. Apoptotic granulocytes retain intracellular contents, such as cytotoxic proteases, thereby preventing tissue damage. Apoptotic granulocytes are subsequently cleared by tissue macrophages (Savill *et al.*, 1989, Savill *et al.*, 1990, Savill *et al.*, 1992).

Eosinophils possess relatively low number of mitochondria and level of expression of cytochrome *c* (Dewson *et al.*, 2001). Although, there are controversial reports on the expression of Bcl- homologous protein in eosinophils, high level of expression of pro-apoptotic protein, Bax, Bcl-XS (Dewson *et al.*, 2001, Druihe *et al.*, 1998) and anti-apoptotic protein Bcl-XL (Druihe *et al.*, 1998), Bcl-2 Dibbert *et al.*, 1998, Plotz *et al.*,

1998) are reported. Dewson *et al.*, (2001) demonstrated co-localisation of Bax and mitochondria in IL-5 deprived condition in caspase independent manner. On the other hand, IL-5 prevents Bax translocation and release of cytochrome *c* and following caspase activation resulting in an anti-apoptoic effect. The on going contradictory debate continues, however, asIL-5 induced expression of anti-apoptotic Bcl-2 (Dewson *et al.*, 1999, Ochiai *et al.*, 1997) may attenuate constitutive apoptosis in eosinophils.

Interestingly other types of cells such as alveolar epithelial cells (Walsh *et al.*, 1999, Sexton *et al.*, 2001) and macrophages have been demonstrated to clear apoptotic eosinophils, which may be beneficial for the removal of eosinophils in airways in asthma.

1.6. NF-κB and apoptosis

1.6.1. NF-κB

NF-κB is a family of transcription factors that regulate genes involved in controlling apoptosis but also mediating much pro-inflammatory gene expression (see review by Baeurerle and Baltimore, 1996, Baldwin, 1996, May and Gosh, 1998). The NF-κB family contains the proteins p65/*Rel* A (Nolan *et al.*, 1991), c-*Rel* (Wilhelmsen *et al.*, 1984) or *Rel* B (Ryseck *et al.*, 1992), p50/ NF-κB1 (Gosh *et al,* 1990, Kieran *et al.*, 1990) or p52/ NF-κB2 (Bours *et al,* 1990, Neri *et al.*, 1990). NF-κB transcriptional activity is controlled by inhibitory proteins, IκB, of which the most important may be IκBα, IκBβ and IκBε. The IκB family contain ankyrin repeat domains (ARD) and share the function of retaining NF-κB in the cytoplasm. IκBα is the major form of IκB in most cells in vertebrates. IκBα is a 37KDa protein that binds to NF-κB in the

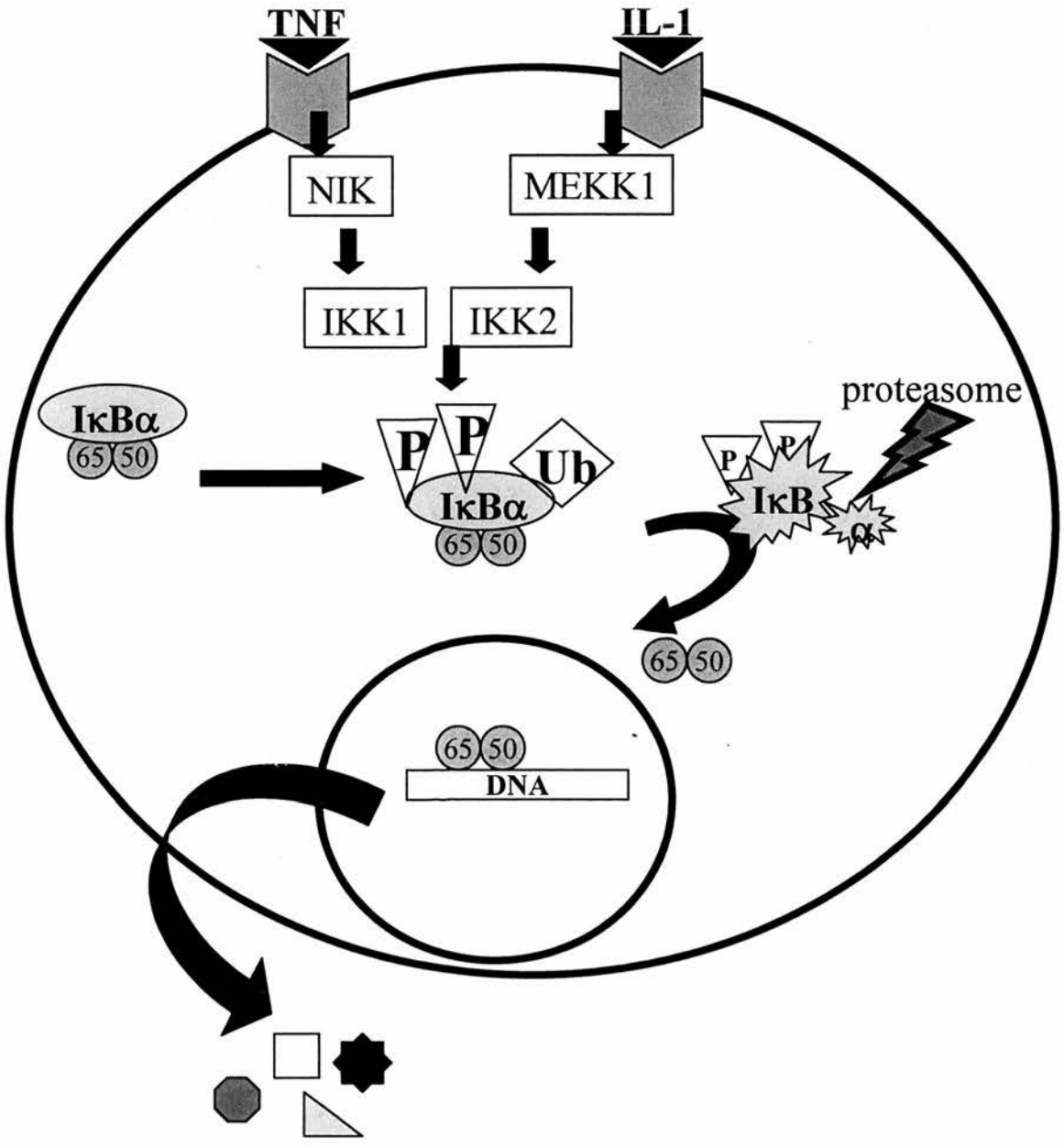


Figure 1-6-1; Schematic representation of components of the NF-κB signal transduction pathway. (Modified from Mercurio and Manning 1999)

cytoplasm. Therefore, NF-κB is prevented from nucleus translocation for gene expression.

NF-κB activation is regulated by the state of IκBα in the cytoplasm. (See figure 1-6-1.) The fate of IκBα is dependent on its phosphorylation and ubiquitination. The pro-inflammatory cytokines TNFα and IL-1β initiate a signalling cascade via their receptors on the cell surface and activate two IκB kinases, IKK-1 (or IKK-α) and IKK-2 (or IKK-β), which lead the phosphorylation on serines 32 and 36 on IκBα (DiDonato *et al,* 1997, Regnier *et al.*, 1997). IKK1 and IKK2 are signal transduction kinases, containing an amino-terminal kinase domain and carboxy-terminal region with two protein interaction motifs, a leucine-zipper and a helix-loop-helix motif (Mercurio *et al.*, 1997). Phophorylated IκBα is selectively ubiquitinated on lysine 21 and 22 by an E3 ubiquitin ligase and finally degraded by the 26S proteasome (Chen *et al.*, 1995). Once IκBα is degraded, NF-κB is free to translocate in the nucleus where it can activate gene expression.

1.6.2. Inhibition of NF-κB for the induction of apoptosis

The pro-inflammatory cytokine TNFα can trigger at least two pathways; a pro-survival pathway via the NF-κB pathway for transcriptional activity and a pro-apoptotic pathway via caspase cascades. There is some evidence demonstrating that TNFα-induced inhibition of NF-κB un-masks the caspase-dependent pro-apoptotic properties of TNFα. Beg *et al.*, (1996) demonstrated that fibroblasts and macrophages from *Rel*A deficient (*Rel*A-/-) mice have reduced viability when these cells were stimulated with TNFα, however *Rel*A +/+ cells were unaffected. Similarly transfection of S32 and S36

mutated IκBα, which is the super-suppressive form of IκBα, into human fibrosarcoma cell lines (Wang *et al.,* 1996), primary mouse and human fibroblasts and Jurkat cells (Van Antwerp *et al.,* 1996), increases TNFα mediated apoptosis. Furthermore, it was demonstrated that disruption of the NF-κB pathway with dominant-negative TRAF2 enhances TNFα-induced apopsosis (Hsu *et al.,* 1996). These results strongly suggest that inhibition of NF-κB enhances TNFα cytotoxity.

A possible explanation for these observations are that NF-κB may produce anti-apoptotic proteins which would protect the cells from the cytotoxic effect of TNFα. These proteins therefore may not only be house keeping proteins but may also be inhibitory proteins preventing apoptosis. For instance, NF-κB upregulates TNFα superfamily receptor complexes, such as TRAF-1and TRAF-2, and the family of inhibitor of apoptosis (IAP), such as XIAP, c-IAP1, c-IAP-2 (Stehlik *et al.,* 1998, Deveraux *et al.,* 1997, Deveraux *et al.,* 1998), which inactivate caspases resulting in survival. TNFα therefore regulates the delicate balance between pro- and anti-apoptotic pathways in cells. Therefore, inhibition of NF-κB plays an important role in the regulation of apoptosis a process that may be applicable regulation of inflammatory cell numbers.

1.6.3. Inflammation and NF-κB

NF-κB upregulates the production of many cytokines including IL-1, IL-2, IL-2R, IL-6, IL-8, G-CSF, GM-CSF and TNFα from various types of cells (reviewed by Baererle and Henkel, 1994). These cytokines not only cause recruitment of inflammatory cells, but can also activate those accumulated inflammatory cells and surrounding cells,

resulting in promotion of the inflammatory process. (See chapter 6 introduction for eosinophils chemoattractant cytokines.) For instance, p50 (-/-) mice are incapable of mounting eosinophilic airway inflammation due to lack of production of IL-5, eotaxin, MIP-1α, MIP-1β (Yang *et al.*, 1998), indicating NF-κB involvement in eosinophil accumulation in the airway. Also, in eosinophils, genes for granule proteins EDN and ECP were upregulated by NFAT-1, which is a sub family of NF-κB (Handen *et al.*, 1997). Therefore, inhibition of NF-κB is one of the primary therapeutic targets for the resolution of inflammation. Some anti-inflammatory drugs, such as aspirin and salicylates (Yin *et al.*, 1998), helenalin (Lyss *et al.*, 1997), curcumin (Jobin *et al.*, 1999), dexamethasone (Ray and Prefontaine, 1994, Caldenhoven *et al.*, 1995), have been shown to suppress NF-κB activation.

Inhibition of NF-κB suppresses production of pro-inflammatory cytokines. On the other hand, inhibition of NF-κB enhances the cytotoxity of TNFα in certain cells. If apoptosis is induced in granulocytes at inflammatory sites by pharmacological reagents, it may offer another approach for the successful resolution of inflammation. In addition, inhibition of NF-κB will result in the suppression of pro-inflammatory cytokine generation another beneficial effect that would be important for successful resolution of inflammation.

1.7. Summary and aims of this thesis

In this thesis, the role of NF-κB activation in the regulation of eosinophil survival and the potential contribution of apoptosis to the massive eosinophil presence found in Hodgkin's disease was investigated.

Chapter 2
Methods and Materials

2.1. - 2.13. Methods and Materials

2.1. Granulocyte Isolation

2.1.1. Granulocyte Isolation

Normal or mildly atopic donor venous blood was collected and anticoagulated {4ml 3.8% sodium citrate/ 36ml blood} and centrifuged (350g, 20 min, room temperature). This gives two layers, an upper layer containing platelet-rich plasma (PRP), and a lower layer containing a mixture of erthyrocytes and leukocytes. The PRP was removed and used to prepare autologous serum in glass tubes by the addition of $CaCl_2$ {22µl of 1 M $CaCl_2$ per 1ml PRP} at 37°C. To sediment the erythrocytes, 5ml of 6% dextran (pre-warmed to 37°C) was added to the pelleted cells and the volume made up to 50ml with 0.9 % saline (pre-warmed to 37°C) and mixed. The cells were allowed to sediment for 30 min at room temperature resulting in formation of two distinguishable layers; a bottom layer containing mainly sedimented erythrocytes and an upper leukocyte-rich layer. The leukocyte-rich layer was aspirated, centrifuged (350g, 6 min) and the supernatant discarded. The resulting leukocyte pellet was resuspended in 2.5ml of 55% isotonic Percoll (9:1 v/v Percoll: 10 x PBS) in 1 x PBS without divalent cations. Discontinous Percoll gradients were prepared by overlaying 2.5ml of 68% Percoll onto 2.5ml of 79% isotonic Percoll. Leukocytes were then resuspended in 55% Percoll and overlayed to form the final layer of the gradient. The gradients were centrifuged (720g, 20 min) and polymorphonuclear cells harvested from the 68%/79% Percoll interface. Mononuclear cells sedimented at the 55%/68% Percoll interface. Purified cells were washed sequentially in PBS without divalent cations twice and cell yield assessed using a haemocytometer. Although the above density gradient centrifugation method does not separate neutrophilic from eosinophilic or basophilic granulocytes, the harvested polymorphonuclear cells generally consisted of <3%

eosinophils, and basophils were rarely seen. Preparations of granulocytes containing >5% eosinophils were used for preparation and study of eosinophils as described in section 2.1.2. Cell viability was assessed by trypan blue exclusion and was routinely >99%.

2.1.2. Eosinophil isolation

Eosinophils were prepared from polymorphonuclear cells, isolated as described above (2.1.1). Separation of eosinophils from neutrophils was achieved through negative selection by immunomagnetic separation using the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr J. Unkeless, Mount Sinai Medical School, New York) and coated sheep anti-mouse IgG-Dynabeads. Anti-CD16-Dynabeads were prepared under sterile conditions by combining anti-CD16 with Dynal M450 sheep anti-mouse dynabeads (typically 20ml supernatant of 3G8 1ml beads). The antibody/bead mixture was rotated at 4°C for at least 20 min to allow antibody binding. The coated beads were then sequentially washed 4 times in PBS without divalent cations (4°C) and the beads retrieved using a stationary contact (3 min) with a magnet (Dynal Magnetic Particle concentrator, MPC-1).

Granulocytes, isolated as in Section 2.1.1. were incubated with washed 3G8-Dynabeads at a bead:granulocyte ratio of 3:2 on a rotary mix at 4°C for 10 min and the beads with attached neutrophils were magnetically separated by stationary contact (3 min) with a magnet. This procedure was repeated once. Purity was assessed by light microscopy of cyto-centrifugated cells, stained with Diff-Quik™. Purified eosinophils (> 98%) were washed and centrifuged (220g, 5 min) twice in PBS without divalent cations, before cell yield was assessed by haemocytometer counts.

2.2. Assessment of Eosinophil Apoptosis

2.2.1. Morphological assay

Eosinophils in Iscove's DMEM supplemented with 10% autologous serum were cultured in Falcon flat-bottomed flexible 96 well plates at 37 °C in a 5% CO_2 atmosphere at 2 x 10^6/ml. Cells were cultured for a set time in the absence or presence of test agents as described in each experiment. Cells were cyto-centrifuged, fixed in methanol, stained with Diff-Quik™ and counted using oil immersion microscopy (x100 objective) to determine the proportion of cells with distinctive apoptotic morphology. At least 500 cells were counted per slide. All experiments were performed at least 3 times and each treatment done in duplicate. The results are expressed as the mean % apoptosis (except experiments performed during Hodgkin's disease study, see 6.2.1.). Trypan blue assay was routinely performed to check the viability of the cells.

2.2.2. Annexin V binding

Stock annexin V was diluted 1:200 with binding buffer {2.5mM CaCl2 in Hank's balance salt solution} and 20µl of 3.0 x 10^6 cells/ml cells were added. Following 10 min incubation at 4°C, samples were fixed by the addition of 100µl of 3% paraformaldehyde in PBS before analysis using an EPICS Profile II.

2.2.3. DNA ladder

Cells (5.0 x 10^6) were washed with PBS and lysed with 500µl of 7M guanidium hydrochloride. Using a Qiagen mini-prep kit, DNA was isolated according to the

manufacturer's instructions. After DNA was isolated, DNA was run on an 1% agarose gel with 1μg/ml EtBr in TBS. Gel was analysed on UV light.

2.3. Western blotting

2.3.1. Preparation of cytoplasmic extracts

Following incubation at 37 °C with the indicated reagents cells were washed with ice cold PBS and the cells lysed at 4 °C for 15 min with Nonidet P-40 (NP-40) lysis buffer {50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 mM β-glyceropyrophosphate, 0.5 % (v/v) Nonidet P-40, 2 mM EDTA, 16.8 mM Na_2HPO_4, 3.2 mM NaH_2PO_4} containing complete protease inhibitor cocktail EDTA free (Boehringer) for eosinophils and {0.5mM ABSEF, 10μg/ml aprotinin, 2mM levamisole, 10μg/ml leupeptin, 1mM sodium orthovanadate, 0.5mM benzamidine, 10μg/ml pepstatinA, 1mM phenanthroline, 1mM PMSF} for neutrophils. The cell fragments were then centrifuged (4300g, 10 min, 4 °C) and the supernatants containing the cytoplasmic extracts collected. The protein loading was controlled by cell number either total protein amount per condition. Each time WB was probed with ponsour staining to determine the equal loading.

2.3.2. Western blot analysis

The cell extracts were diluted with 3 x sample buffer {5.7% (wt/v) SDS, 14% β-mercaptoethanol, 150mM Tris HCl, pH 6.7; 30% (v/v) glycerol} and heated at 95°C for 3 min before running on 10 % (wt/v) SDS -polyacrylamide gel. Electophoretically separated proteins were transferred on PVDF microporous membranes. Non-specific binding was blocked with PBS containing 5% non fat dry milk and 0.1% Tween 20 and probed with the indicated antibodies. As a positive control, protein extracted from

Jurkat cell extract was also run on the gels. Immunodetected proteins were visualised by ECL™ detection reagents.

2.4. Immunohistochemistry

2.4.1. Staining for immunohistochemistry

Eosinophils (2.5 x 10^6/ml) were incubated in Iscove's DMEM containing 5% FCS for 2 hours at 37°C before treatment with the indicated agents and time. The cells were cyto-centrifuged, air dried for 10 min and then fixed with 4% (w/v) p-formaldehyde/PBS for 10 min and washed 3 times with PBS. The cells were permeabilized and non-specific binding was blocked in buffer containing 0.2% (w/v) Triton X-100 in DAKO Protein Block Serum Free buffer at room temp for 30 min. The cells were incubated with rabbit polyclonal p65 antibody (1:100 dilution) in DAKO Antibody Diluent with 0.2 % Triton X-100 for 1 hour. The cells were washed 3 times in DAKO Antibody Diluent with 0.2 % Triton X-100 and then incubated (1:100 dilution) with Alexa™ 488 (goat anti-mouse) IgG antibody for 1 hour in DAKO Antibody Diluent with 0.2 % Triton X-100 then washed 3 times in the same buffer. The nucleus was stained with 0.1% propidium iodide with 2% RNAse in PBS at 37°C for 30 min followed by washing (x 3) with PBS before applying a glass coverslip.

2.4.2. Confocal microscopy and image analysis

Images are from single confocal sections, 0.3μm thick, taken through the center of the nucleus, on a Leica TSC 4D confocal microscope using a 63x objective lens. p65 nuclear translocation was determined with a Leica DMRB microscope using a 40 x NA

0.7 objective lens and Kappa integrating monochrome CCD camera. Image analysis was integrated with image capture as described before (Dptala *et al.*, 1998). The image analyzer captures an image of the green p65 fluorescence and using an automated macro-program developed for this application on a Leica Q550IW image analyser and uses the binary images of the propidium iodide staining as a mask to measure only the p65 fluorescence of the nuclei. The image analyser then detects the green fluorescence (488nm excitation, 530nm emission) and subtracts the binary image of the red fluorescence (570nm excitation, 620nm emission) to give a binary mask for the cytoplasm.

2.5. ELISA for IL-8 Measurement

The ELISA (enzyme-linked immunosorbent assay) was performed using IL-8 recombinant protein as a standard and IgG anti-human IL-8 according to the manufacturer's instructions (R & D systems).

Briefly, 100µl of IL-8 mAB (4µg/ml in TBS) per well on a flat bottom EIA/RIA Plate and left over night. The plate was washed three times with TBS 1% Tween 20, and blocked with TBS 1%BSA for 1 hour. After blocking, samples were incubated with standard samples (typically concentrations of 20ng/ml, 10ng/ml. 5ng/ml etc.,) for 2 hours. The plate was then washed three times with TBS 1% Tween 20, and incubated with detection antibody {biotinylated 20ng/ml in sample buffer; 0.1% BSA in TBS} for 2 hours. The plate was then washed three times with TBS 1% Tween 20, and incubated with 0.005% streptavidin-HRP in sample buffer for 20 min. The plate was then washed three times with TBS 1% Tween 20, and incubated with TMB 100µg/ml in 100mM sodium acetate-citrate pH 4.9 with 0.015 % H_2O_2 for 20 min in the dark.

Then, 1M H_2SO_4 is added to stop the reaction. The plate was read on a Microplate Reader 450 (BioRad Laboratories) at a wave length of 595nm.

2.6. Cloning TAT-IκBα

pcDNA

pcDNA IκBαWT and pcDNA IκBα32,36 were gifts from Prof Hay, University of St Andrews. pcDNA IκBαWT is full length wild type IκBα, pcDNA IκBα32,36 is the full length IκBα but mutated at amino acid 32 and 36 serine to alanine. pGEX2T, containing glutathione S-transferase (GST) gene, was obtained from Pharmacia (See figure 5-2-1-A for plasmid map).

E.coli

E.coli DH5α (genotype; Φ80d*lac*ZDM15, *rec*A1, *end* A1 *gyr* A96, *thi*-1, *hsd* R17 (r, -, mk+), *sup* E44, *rel*A1, *deo*R, D(*lac*ZYA-*arg*F)U169) was used for mainly DNA preparation.

E.coli B834 (F-,*omp*T,*hsd*S_B, (r_B-,m_B-)*dcm*,*gal*) was used for protein purification.

TAT

amino acid; YGRKKRRQRRR (Nagahara *et al.*, 1999)

TAT oligoenucleotides, containing BarmH1 cut sites, were obtained from MWG-Biotech, phosphorylated at 5'.

TAT top; 5' *GA TCT* TAT GGC CGC AAA AAA CGT CGC CAG CGT CGC CGT *G* 3'

TAT bottom; 3' A ATA CCG GCG TTT TTT GCA GEG GTC GCA GCG GCA CCT AG 5'

2.6.1. Insertion of pcDNA IκBαWT and pcDNA IκBα32,36 into pGEX2T

Aim; to produce pGEX2T containing GST-IκBα or GST-IκBα32,36 gene

Isolation of pcDNA and cut off pGEX2T

pGEX2T contains high level expression of genes as fusions with GST gene from *Schitsoma japonicum*, which forms an affinity tail on the protein products of genes inserted into the multiple cloning site. pGEX2T was digested by BamH1 {5 μg DNA, 5μl 10 x Buffer E (Promega), 2 μl BarmH1 (Promega)}, pcDNA IκBαWT and pcDNA IκBα32,36 were digested by EcoRI {5μg DNA, 5μl 10 x Buffer (Promega), 2μl EcoRI (Promega)} and BamHI at 37°C for 3 hours. Digested DNA was run on a 1% agarose gel with 1μg/ml EtBr. Efficiently digested pcDNA was extracted from the gel under UV light and purified with using a Qiagen Gel Extraction Kit, according to the manufacturers instructions. Final concentration of DNA was determined for the ligation.

2.6.2. Ligation and insertion into DH5α

Ligation was set up for typically, x1, x3, x6, x9, x20, x30 of pcDNA against digested pGEX2T for several hours at 20 C {100ng pGEX2T DNA, 1μl 10x ligase buffer (Promega), 0.5μl T4 DNA ligase (Promega), 6.5μl H_2O}. DH5α, competent cells, were heat shocked at 42 C for 3 min for DNA insertion {100μl of cells (from the stock of the cells OD600nm=0.6) to 5μl of ligated DNA}. Cells were cultured with {400μl Luria-Bertani (LB), 20mM glucose} on a shaker for 37 C for 1 hour. Then the cells

were seeded onto the plates {LB, 50μg/ml Ampicillin (AMP), 0.7% agar} and incubated for 37 C for several hours.

2.6.3. Selection of the colony by PCR and DNA sequencing

After the incubation several colonies were selected for 5ml culture {LB, 50μg/ml AMP} growing for several hours on the shaker at 37 C. After growing the cells, mini prep (Qiagen) was perfomed to determine for PCR and DNA sequence, and a small volume of protein purification was performed to determine the colony which contains the appropriate DNA.

2.6.4. PCR

Primer; GC rich E14A down; GCG GGG GCC CGC CAT GGC CCA (Professor Hay, University of St Andrews).

PCR was proceeded for 35 cycles, 94°C 5min, 94°C 5min, 55°C 5min, 72°C 3min, 72°C 10min.

2.6.5. DNA sequence

DNA sequence was determined using an ABI PRISM™ 377 DNA Seqeuencer by Alex Huston at the DNA sequence Unit, St Andrews University. (See chapter 5, result section figure 5-2-2 for a successful GST-TAT-IκBα insertion.)

2.6.6. Protein purification for small volumes

See protein purification session (2.7.) as follows. A small volume of the cells, such as in 5ml medium culture, was used.

2.6.7. Selection of the colony

The right colony was selected depending on the results obtained from PCR, DNA sequence and protein purification procedures. If it was necessary maxi prep (Qiagen) was performed.

2.6.8. Insertion of double stranded TAT into pGEX2T containing IκBαWT or IκBα32, 36

Aim; to produce pGEX2T containing GST-TAT-IκBα or GST-TAT-IκBα 32,36 gene

Anealling single strand TAT oligonucleotides into double stranded DNA.

Single stranded TAT oligonucleotides were anealled with 10x hibridisation buffer {1M NaCl, 100mM Tris pH 8.0, 10mM EDTA} for 50pM top and bottom oligonucleotides at 100°C for 3 min then allowed to cool down to room temperature. Double stranded TAT DNA was inserted into pGEX2T containing IκBα or IκBα32,36 as described above (see 2.6.1. and 2.6.2.).

2.7. Protein Purification

2.7.1. Protein expression in B834

The DNA containing GST-TAT-IκBα or GST-TAT-IκBα32,36 were transformed into chemically competent *E.Coli* B834 by heat shock as described above. After several hours incubation on an agarose plate, a few colonies were selected for growing in 500ml cultures {LB, 50μg/ml AMP}. (DNA sequence or PCR were obtained.) B834 cells were cultured on the shaker at 20-37 C, depending on the speed of their growth. When OD600nm was between 0.6 and 0.8, 0.5mM IPTG was added. After 2 to 3 hours of growth the cells on the shaker at 20 C cells were harvested . Some cells were kept in a glycerol stock (10%) at -70 C. The remaining the cells were lysed with Buffer A

{50mM Tris pH8.0, 500mM NaCl, 0.1% β-mercaptoethanole} at 4°C (all the process must be handled at 4°C after this process except enzyme digestion) and sonicated until the medium colour gets clear. 1% Triton-X was added and centrifuged for 5 min at 6000g. The supernatant was used as cell lysate.

2.7.2. Protein purification through column

GST-beads in the column was washed with Buffer A {50mM Tris pH8.0, 500mM NaCl, 0.1% β-mercaptoethanole} before applying the cellular lysate to the column (this allows GST-TAT-IκBα binding to GST beads in the column). 10mM glutathione was applied to the column and 1ml flow-through fraction was collected. The peak protein fraction was measured using the Bradford assay at 595nm. (This peak fraction should contain GST-TAT-IκBα) Thrombin 2 units/mg protein used to digest the peak fraction (to cut off GST from TAT-IκBα), for 1 hour at room temperature. A protein gel (10% acrylamide SDS gel) was run to check the efficiency of digestion by thrombin, stained with comassie blue. The Q-sepharose beads in the column was washed with Buffer B {50mM Tris pH8.0, 250mM NaCl, 0.1% β-mercaphtoethanol}. Thrombin digested protein fraction (TAT-IκBα) was diluted with 250mM NaCl with Buffer C {50mM Tris pH8.0, 0.1% β-mercaptoethanol} for application to the Q-sepharose column. (This allows TAT-IκBα binding to Q-sepharose beads and to elute away GST.) Buffer D {50mM Tris pH8.0, 625mM NaCl, 0.1% β-mercaphtoethanol} was applied and flow-through was collected, and the protein measured at 595nm using the Bradford assay (Peak fraction should contain TAT-IκBα). TAT-IκBα was examined by 10% SDS gel and mass spectrometry by slow crystalisation (Dr Botting, St Andrews, see fig5-2-3) were performed to be confirm correct protein determination. Protein was kept in 10% glycerol at -70°C.

2.8. Denaturing the protein

TAT-IκBα or TAT-IκBα32,36 protein (300- 500μg) were dialysed with 8M urea against DMEM for 3 hours. Medium was changed every hour. After the dialysis 20mM HEPES was added and the protein kept at -70°C with 10% glycerol.

2.9. Luciferase assay

Luciferase-reporter-gene-inserted cells (Arenzana-Seisdedos *et al.*, 1997) were used to examine the expression of NF-κB following treatment with TAT-IκBαWT or TAT-IκBα 32,36.

HeLa cells; 57A (Professor Hay, University of St Andrews), maintained in 10% FCS in DMEM

Epithelial cells; A549 (Professor Hay, University of St Andrews), maintained in 10% FCS in DMEM

Jurkat cells; α5.1 (Professor Hay, University of St Andrews), maintained in 10% FCS in RPMI 1640 (Gibco)

Six-well plates were used to culture adherent cells. Cells were stimulated with various stimuli and 5-6 hours later the cells were washed with ice cold PBS and harvested with lysis buffer {25mM Tris pH 7.8, 8mM MgCl2, 1mM DTT, 1% TritonX-100, 15% glycerol}. Cellular lysate (100μl) was read using a luminometer with injecting 100 μl luciferase buffer {1mM ATP, 0.25mM luciferin, 1% BSA made up in lysis buffer}. Protein amount was measured using the Bradford assay to determine luciferase activity/ protein amount (RLU/mg).

2.10. Labelling TAT with FITC

Fluorescent Isothiocyanate (FITC) was incubated, in a ratio of 40-80μg per mg of protein, with TAT-IκBα, recombinant IκBα, GST-TAT for 1 hour at room temperature, overnight at 4°C in the dark. After the incubation, protein was dialysed against buffer {10mM Tris, 150mM NaCl, pH8.2}, over night at 4°C with four changes of buffer. After dialysis protein concentration was measured.

2.11. Gel shift assay

After cells were lysed to obtain cytoplasmic extracts, the nuclei were washed twice with cytoplasm lysis buffer {50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 mM β-glyceropyrophosphate, 0.5 % (v/v) Nonidet P-40, 2 mM EDTA, 16.8 mM Na_2HPO_4, 3.2 mM NaH_2PO_4, containing complete protease inhibitor cocktail EDTA free (Boehringer)}. The nuclei were then lysed with ice cold nucleus lysis buffer {300mM NaCl, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 mM β-glyceropyrophosphate, 0.5 % (v/v) Nonidet P-40, 2 mM EDTA, 16.8 mM Na_2HPO_4, 3.2 mM NaH_2PO_4, containing complete protease inhibitor cocktail EDTA free} for 15 min on ice and centrifuged (23,000g for 10 min at 4°C). The nuclear extract was obtained and its protein concentration was measured.

10μg of nucleus extract was incubated with 18μl binding buffer {25mM Hepes, 1mM EGTA, 3.5mM Spermidine, 6mM $MgCl_2$, 0.15% NP-40, 10% glycerol}, 2μl {100mM DTT, 1μl 10mg/ml BSA, 1μl 0.5mg/ml poly dAT, 1μl 10mg/ml BSA, 1μl 0.5mg/ml poly dGC} for 15 min on ice. Then 1μl of ^{33}P (10,000cpm, approx) labelled DNA

containing an NF-κB recognition site (see below for various oligonucleotides) was added for 15 min at room temperature before being assayed by electrophoresis on 6% non-denaturing polyacrylamide gels. After electrophoresis, gels were dried on 3MM paper (Whatman) and visualised using a phosphoimager.

The NF-κB oligonucleotides were as shown below;

Oligonucleotides;

16bp WT κB;

5' CTG **GGGACTTTCC** AGG 3'
3' GAC **CCCTGAAAGG** TCC 5'

HIV-R;

5' GATCTG **GGGACTTTCC** AGG 3'
3' AC **CCCTGAAAGG** TCCCTAG

Promega;

5' AGT TGA **GGG GAC TTT CC**C AGG C 3'
3' TCA ACT **CCC CTG AAA GG**G TCC G 5'

2.12. Hodgkin's disease studies

2.12.1. Hodgkin-Reed-Sternberg (HRS) cell lines

Six different HRS cell lines (L428, L540, L591, KMH2, HDLM2, HDMYZ) were maintained in RPMI1640 with heat inactivated 10% Foetal calf serum (FCS), except HDMYZ in RPMI 1640 with heat inactivated 20% FCS. These suspension cells were split to $0.5x10^6$ cells/ml density every 2-3 days and kept at 37°C in a 5% CO_2 atmosphere.

2.12.2. Supernatant study

HRS cells were centrifuged at 350g for 5 min and supernatants were taken for further incubation with eosinophils or neutrophils. Supernatant was collected from different passages on several separate occasions. Typically 10% of supernatant in final a volume during granulocytes culture was investigated (see 6.2.1. for more details).

2.12.3. Isolation of substances by molecular weight from the supernatant from HRS cells.

Centriplus®, centrifugal filter devices, (Millipore) was used to separate the molecules from HRS supernatants with molecular weight 10KDa, 30KDa, 50KDa. Centrifugation was proceeded according to the manufacturer instructions.

2.13. Statistical Analysis

Statistics analysis was performed by Newmans-Kuels procedure. The row value was calculated for mean value where standard error of the mean was taken to evaluate the statistical significance. From standard deviation (defied as statistic that communicates the average of the deviation of the scores from the mean in a set of data, computed by obtaining the square root of the vatienc.) and the standard error of the mean difference from each condition were taken to evaluate the statistical significance. These values were obtained using T-test ($p<0.05$) and value was determined its significance.

2.14. Materials

The following reagents were kindly donated as gifts:

- murine anti-neutrophil antibody 3G8 from Dr J. Unkeless, Mount Sinai Medical School, New York,
- anti-TAT AB from Dr Eric Vives, Institute de Genetique Moleculaire de Montpellier, Montpellier, France
- pcDNA IκBαWT and pcDNA IκBα32,36, anti-IκBα antibody 10B (Jaffray *et al.*, 1995) and AP were gifts from Professor Hay, University of St Andrews.

Further materials were purchased from the following companies:

5 Prime->3 Prime, Inc: Pancreatic Rnase A (DNase-free) (5-888777)

Affiniti Research Products Ltd, Mamhead, Exeter, UK: z-VAD-fmk methyl ester,

Agar Scientific LTD: PAP PEN (L4197)

ALEXIS Biochemicals : MG-132 (N-cbz-Leu-Leu-leucinal)

Amersham Pharmacia Biotech, UK Ltd, Buckinghamshire, UK: horse radish peroxidase-conjugated donkey anti-rabbit secondary antibody, [gamma-^{32}P], [gamma-^{33}P] ATP (3000 Ci/mM), Ultrapure dNTP set 2'-deoxynucleoside5'-triphosphate (27-2035), Percoll, Poly di/dc. Poly di/dc 5U (27-7880), ECL detection reagents (RPN

2105, RPN 3004), dNTP (27-2080-01), dATP (27-2050-01), dCTP (27-2060-01), dGTP (27-2070-01)

Baxter Healthcare Ltd, Baillieston, Glasgow, Scotland, UK: Diff Quik™ stain. Solution I (Eosin G in phosphate buffer, pH 6.0), Solution II (Thiazine blue in phosphate buffer; pH 6.0), saline solution 0.9% (sterile).

Boehringer Mannheim, Germany: Annexin-V-FLUOS

Cambridge Bioscience, Cambridge, UK: Alexa 488 goat anti-rabbit (A-11034)

Cell Signalling Technology, Beverly, MA, UK: polyclonal rabbit anti-IκBα antibody

Costar: EIA/RIA Plate flat bottom high binding (3590) for ELISA

DAKO Ltd Cambridgeshire, UK: Horse radish peroxidase-conjugated, goat anti-mouse, Anti-mouse AB; peroxidase-conjugated goat anti-mouse IgG (P0447), anti-sheep AB, peroxidase-conjugated rabbit anti-sheep immunoglobulins (P 0163), DAKO® antibody diluent (S0809), DAKO® Antibody Diluent (S080981), DAKO® protein block serum-free (X0909)

DAKO® Protein Block Serum-Free (X090980); DAKO

Dynal UK, Wirral, UK: Dynabeads M-450 sheep anti-mouse IgG. Supplied as 4 x 10^8 beads/ml in PBS pH 7.4 with 0.1% human serum albumin and 0.2% sodium azide.

Euronenetics: Anti-CD16 mAB, 5D2 clone (M1389)

Genzyme Diagnostics, Kent, UK: GM-CSF (1000 U/ml in PBS) was stored at -70°C.

Gibco Life Technologies, Paisley, Scotland, UK: Iscove's Dulbecco's modified Eagles medium, without supplements with L-glutamine (Iscove's MDM); Hanks Balanced Salt Solution (HBSS); RPMI1640; foetus calf serum; culture supplements penicillin (50 U/ml)/streptomycin (50 U/ml); L-glutamine (200 mM); and 30 % (w/v) acrylamide/bis solution.

Life Technologies : BenchMark™ Prestained protein ladder (10748-010)

Martindale Pharmaceuticals Ltd, Romford, UK: calcium chloride.

Melford Lab Ltd; IPTG

Millipore: CENTRIPLUS® YM-10, CENTRIPLUS® YM-30, CENTRIPLUS® YM-50; Immobilon-P PVDF

Organon Laboratories Ltd, Cambridge, UK: Dexamethasone.

Phoenix Pharmaceuticals Ltd, Gloucestershire, UK: Sodium citrate solution (3.8%).

Piece chemical company: BCA Protein Assay Reagents (Kit) (0023223)

Promega Corporation, Southampton, UK: SignaTECT™ cAMP-Dependent Protein Kinase (PKA) Assay System, T4 DNA Ligase; T4 Polynucleotide Kinase (M4101), NF-κB consensus ologonucleotide, DNA Polymerase I Large (Klenow) Fragment (M2201),

QUIAGEN: QIAGEN® Plasmid Kits (12143, 12145)

R&D Systems Europe Ltd, Oxon, UK: TNFα (stock solution 10μg/ml); mouse IgG_1 anti-human IL-8 monoclonal antibody (stock solution 500μg/ml), biotinylated anti human IL-8 polyclonal antibody (stock solution 50μg/ml), human IL-8, Anti-IL-13 AB; monoclonal anti-human neutralizing IL-13 antibody (MAB213), TNFα; TNFβ, IL-5, GM-CSF

Roche Diagnostics GmbH: Complete mini-EDTA free protease inhibitors (1836170)

Santa Cruz Biotechnology, Inc: Anti-IκBα antibody,C21, Anti-IκB-β antibody, Anti-IKK1 antibody, IKKα(B-8), (sc-7606), Anti-p50 antibody, NF-κB p50 H-119 (sc-7178), Anti-p50 antibody, NF-κB p50 N-19 (sc-1191), Anti-p65 antibody, NF-κB p65 H-286 (sc-7151), Anti-SUMO-1 antibody,C-9, (sc-6375)

Shandon, Pittsburgh, PA, USA: Shandon Filter Cards

Sigma; other reagents not on this list purchased by Sigma

Transduction Laboratories, San Diego, California, USA: Rabbit anti-human caspase-3 polyclonal antibody,

Chapter 3
The methodology to investigate the function of NF-κB in granulocytes.

3.1. Introduction

The study of specific intracellular proteins in granulocytes, by biochemical or molecular approaches has some technical difficulties due to the high amount of proteases and their short lives. (The half life of neutrophils and eosinophils are 12 hours and 2-4 days, respectively.) Of these cells in fact, compared to studies in other cell lines, fewer biochemical and molecular studies have been demonstrated. For example in the NF-κB area, Cassatella's group has demonstrated IκBα degradation and NF-κB activation in neutrophils (McDonald *et al.*, 1997, McDonald *et al.*, 1998a, McDonald *et al* 1998b, McDonald and Cassatella 1997). Our study (Ward *et al,* 1999) demonstrated the importance of NF-κB in neutrophil apoptosis, and others have demonstrated NF-κB gel shift assay for neutrophil adhesion molecules (Rahman *et al.,* 1999), phagocytosis (Vollebregt *et al.*, 1998) and activation (Shenkar and Abraham 1999). In eosinophils Yamashita *et al.*, (1999) showed for the first time the immunofluorescent staining of p50, and our study (Fujihara *et al.,* 2002, see chapter 4) demonstrated the expression of IκBα and p65 in the cytoplasm and nucleus. Despite the huge therapeutic interest, these are the only NF-κB related published reports in granulocytes (in both neutrophils and eosinophils).

In this chapter the methodology used to study NF-κB in neutrophils and eosinophils is investigated. In neutrophils the usage of different antibodies, culture conditions and expression of IκBα are shown. The expression of IκBα in neutrophils is extension of the study we published recently (Ward *et al.*, 1999) to investigate the effect of various NF-κB inhibitors on neutrophil apoptosis. On the other hand, in eosinophils, expression of p50, p65, IκBα and IκBβ are shown in this chapter. Then

in the following chapters (4, 5 and 6), the role of NF-κB in apoptosis and survival in eosinophils is thoroughly investigated.

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3.2. Results;

3.2. Morphological assessment of granulocyte apoptosis

As described in the methods chapter (see 2.1.1.) neutrophils or eosinophils were freshly isolated. *In vitro* granulocytes undergo spontaneous apoptosis. Typical half life of neutrophils is 12 hours, whereas in eosinophils it is an average 1 - 3 days, but is dependent on the donor*. Figure 4-5-A and B in chapter 4 shows typical apoptotic eosinophils, which were cultured without any stimulus for 4 (4-5-A) and 30 hours (4-5-B) and stained with Diff Quick (For typical apoptotic neutrophils, see figure 3 in Appendix, Ward *et al.*, 1999). Apoptotic eosinophils clearly exhibit condensed nuclei and shrinkage of cytoplasm. Furthermore, another apoptosis marker, Annexin V binding and eosinophils apoptosis is shown in chapter 4, figure 4-2-5C.

*However, it has been observed that some eosinophils from atopic donor (this experiment was performed in GlaxoSK) survived up to 7-10 days without any stimuli *in vitro*. This suggest that eosinophils from some individuals may have more resistance towards apoptosis but survive longer for some reason, perhaps high cellular metabolism, such as high activity of survival pathway, protein synthesis and inhibition of apoptosis pathway. It is more likely that atopic patients or allergy suffers may have more resistance towards ongoing apoptosis. However, the precise

correlation studies has not yet be performed. As next project, apoptosis rate, peripheral blood eosinophil count, level of IgE in peripheral blood, if asthmatic the measurement of FEV_1 and PC_{20} to methacholine, adenosine monophosphate, histamine challenge, allergy symptom scores etc., should be studied. Please see 5.5.3. for the possibilities of different responses in donor dependent manner to TAT-IκBα in apoptosis rate.

3.3. The methodology and study on NF-κB in neutrophils.

Molecular or biochemical experiments using neutrophils *in vitro* involves some technical difficulties that may not arise in cell line work. This is not only because of the neutrophil's short life span, but also that they contain high quantities of granules with proteases that may disturb the target cellular contents during the isolation process. To obtain the target cellular contents, in this case IκBα from cytoplasm extract, various methods have been attempted as follows.

3.3.1. Protease inhibitors

Strong protease inhibitors are required for the isolation of cellular contents from neutrophils due to the high amount of proteases contents in granules. Cassatella *et al.,* (1998) have suggested the usage of Diisopropyl Fluorophosphate (DFP) for neutrophilic lysate preparation for Western blotting and gel shift assay. DFP is a strong cysteine inhibitor but also highly neurotoxic and required extra caution to handle in the laboratory. We (kindly with the help of Dr James Pryde, CIR, University of Edinburgh) had tried and the results were clear that DFP blocked protease activities (data not shown here, but shown in Pryde *et al.,* 2000) and obtained the target intracellular protein without any trace of degradation or

disruption by proteases. However, in practical terms, it was impractical to continue this procedure for each experiment with extra safety equipment, e.g. wearing gas masks, and concluded that the strong cocktails of protease inhibitors, which can be used normally in the laboratory, were used instead. The effect of the cocktail of protease inhibitors was, however, limited resulting in the occasional failure of isolation of the target proteins due to proteolysis in neutrophils.

3.3.2. Isolation of the target proteins for Western blotting analysis.

To obtain the cellular protein, neutrophils can be lysed in various ways; the standard NP-40 lysis methods, homogenisation (Pryde *et al.*, 2000) and nitrogen cavitation methods (Cassatella *et al.*, 1998b). Homogenisation and a series of centrifugations can isolate various neutrophilic contents (e.g., cell membrane, granules, mitochondria etc.,) and would be the most favourable method to isolate neutrophilic contents without disturbing granule contents. Although, homogenisation may be the best option for isolation, it requires a substantial amount of neutrophils. Therefore, for my experiments, it was concluded that this was not the most suitable method due to lack of the number of cells. In addition, due to a lack of equipment, we did not have a chance to try nitrogen cavitation methods.

As described in the methods section, in my experiments, NP-40 lysis methods, which disrupt the cellular membrane resulting in a burst of the cellular contents, was used for both neutrophil and eosinophil work. This method may disturb the granule contents, but would be controlled by the strong protease inhibitors. The NP-40 method with strong protease inhibitors may not be favourable for the isolation of neutrophilic contents. However, considering the practical reasons and cell numbers,

it was concluded to be an optimal method for the following investigation for the IκBα expression in neutrophils.

Summary

- For practical reasons use of a strong protease cocktail and NP-40 lysis method were used for the following neutrophil and eosinophil experiments.

3.4. Various anti IκBα antibodies

To determine the expression of IκBα by Western Blotting (WB) analysis, various anti- IκBα antibodies were tested in neutrophil experiments. The characterisation of those antibodies are as shown below in table 3-4. 'IκBα' by New England Bio Lab (see 3.13.) were used for eosinophil experiments as shown in the following sections.

name	clone	animal	recognition sites	supplier or gift from
10B	mono-clonal	mouse	a.a.24-48*	Prof Hay, University of St Andrews
AP	poly-clonal	sheep	a whole IκBα	Prof Hay, University of St Andrews
C-21	poly	rabbit	a.a. 297-317	Santa Cruz
IκBα	poly	rabbit	a.a. 27-38	New England Bio Lab

Table 3-4; Various anti-IκBα antibodies have different recognition sites on IκBα. *Jaffary *et al.*, 1995.

3.5. Expression of IκBα from neutrophils cultured in PBS or medium containing 10% autologous serum.

In the resting state of the cells, the transcription factors NF-κB are held by IκBα in the cytoplasm for the prevention of translocation into the nucleus for the following

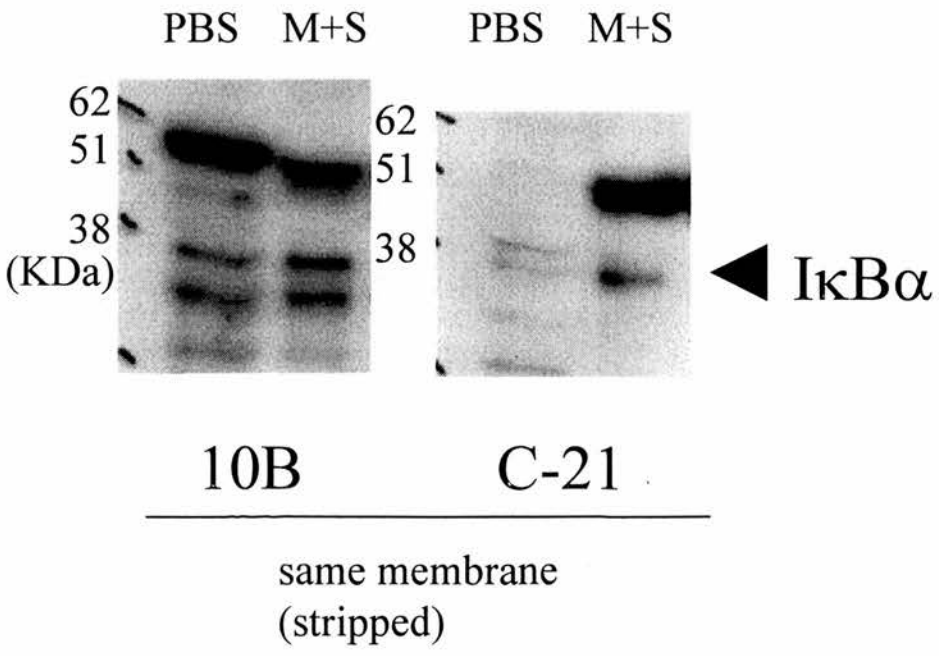


Figure 3-5; Expression of IκBα from neutrophils cultured in PBS or medium containing 10% autologous serum. Purified neutrophils (5.0 x 10^6 cells/ ml) cultured in PBS or Iscove's DMEM containing 10% autologous serum for 30 minutes. Cells were lysed for cytoplasmic extract amd examined by WB. Different anti-IκBα antibodies, 10B and C-21 are used to determine the expression. IκBα is usually expressed as 37KDa.

transcription activities (see introduction 1.6.1. and figure 1-6-1). Upon certain stimuli, such as TNFα, LPS and IL-1, IκBα is phosphorylated and then degraded by 26S proteasome and result in the release of NF-κB into the nucleus. To investigate the IκBα expression in the cytoplasm as an index to measure NF-κB activation (i.e., the degradation of IκBα indicates NF-κB translocation into the nucleus). The expression of IκBα in neutrophils was examined by WB analysis.

Figure 3-5 shows IκBα expression in neutrophils. Neutrophils were cultured in PBS or Iscove's medium containing 10% autologous serum (M+S) for 30 minutes and lysed for cytoplasmic extract for WB analysis. The membrane was probed with 10B antibody first. Then it was stripped and probed with C-21 antibody. The anti-IκBα antibody 10B (monoclonal, recognition site a.a. 24-48) recognises three different bands (55K, 37K and 30KDa) in both PBS and M+S. In contrast anti-IκBα antibody C-21 (polyclonal, recognition site is a.a. 267-317) recognised only two bands (55K and 37KDa) in M+S condition, but not in PBS, although it was the same membrane.

Another anti-IκBα antibody, AP (polyclonal, recognises a whole length of IκBα), had also recognised three bands the same as 10B (see figure 3-9). The different band expression by different antibodies are not fully understood. However, antibody AP and 10B do recognise the same bands although their recognition sites were different. Therefore, it was concluded that AP and 10B were to be used for the following WB analysis but not C-21. (Because C-21 recognise the different bands.)

The three bands (55K, 37K and 30KDa) were repeatedly detected in the following neutrophil WB. Ordinary IκBα in many cells is 37KDa, which is indicated as 'IκBα'

in all the figures in this chapter. However, the mechanisms and functions of 55K and 30KDa are yet to be elucidated. More details are discussed in 3.10.and discussion 3.16.

Summary

- The antibodies 10B and AP (also see figure 3-8) detect the same three bands (55K, 37K and 30KDa) in neutrophils.
- It was concluded that 10B would be used for the following neutrophil WB experiment.
- C-21 recognises only two bands (55K and 37K) in medium containing serum.
- C-21 did not recognise any bands from PBS cultured cells.

3.6. Expression of IκBα from neutrophils cultured in medium with or without 10% autologous serum.

It has been noted that IκBα expression in neutrophils by WB is generally better in the cells cultured in medium containing 10% autologous serum than ones without serum or in PBS. Figure 3-6 shows the IκBα expression from neutrophils cultured in the medium with or without 10% autologous serum. Apart from 60 minutes, all other time points showed better IκBα expression from the cells cultured in serum containing medium. The mechanism of the better signal on WB is not known. This could be because of activation of IκBα in the cytoplasm in PBS condition either experimental artefact that IκBα protein may have been degraded as proteolysis

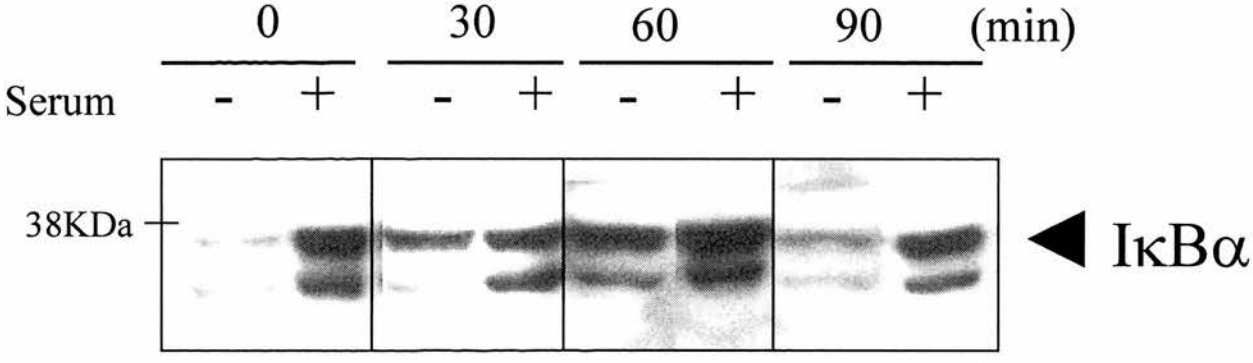


Figure 3-6; Expression of IκBα from neutrophils cultured in medium with or without 10% autologous serum. Purified neutrophils (5.0×10^6 cells/ ml), which had been cultured in Iscove's DMEM with or without 10% autologous serum for the time indicated. Cells were lysed for cytoplasmic extract and examined by WB. Anti-IκBα antibodies (10B) are used to determine the expression.

during the separation of lysate. To determine the real answer, direct measurement of NF-κB activation is required as a future project.

Autologous serum contains various substances which may influence various intracellular factors including protease activities. This may affect the preparation process of obtaining lysate (e.g., inhibition of protease activities) or some protection against proteolytic problems (See discussion 3.16.)

In order to gain better signals of IκBα expression by WB, it was concluded that neutrophils would be cultured in medium containing 10% autologous serum.

Summary

- Neutrophils cultured in medium containing 10% autologous serum demonstrate better IκBα expression by WB.
- Further experiments on neutrophils were performed with medium containing 10% autologous serum.

3.7. 55KDa IκBα is not SUMO-IκBα complex in neutrophil cytoplasm.

As shown in figure 3-5, three bands of 'IκBα' in neutrophils were expressed by WB. Those three bands of IκBα were recognised by anti-IκBα antibody 10B and AP. So far this pattern has not been observed or reported in other cell types, such as HeLa, A549 and also in eosinophils using the same isolation methods. Usually IκBα is detected as one band at 37KDa in many cells.

Recently, it has been shown that SUMO (SUMO; small ubiquitin-like modifier) linked IκBα is expressed in the cytoplasm at about 55KDa by WB (Desterro et al., 1998) in HeLa cells. When IκBα is modified by SUMO-1, SUMO-IκBα complex is resistant from degradation by the proteasome (Desterro et al., 1998), resulting in the inhibition of NF-κB.

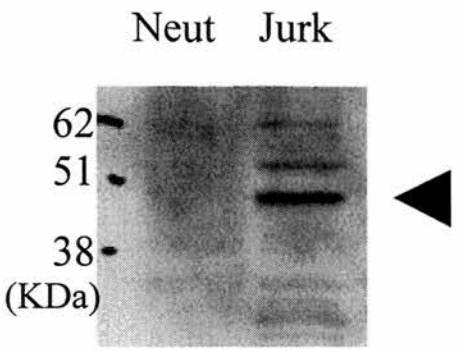
In neutrophils this 55KDa band is detected commonly. Thus, to investigate if 55KDa band is due to SUMO-IκBα complex, neutrophil lysate on WB was to assessed by anti-SUMO antibody. The result is shown in figure 3-7. In Jurkat cells the 55KDa band is recognised as SUMO positive, possibly a complex of SUMO-IκBα, but not in neutrophils. Therefore, it was concluded that this neutrophilic 55KDa band is not due to SUMO-IκBα.

Summary

- Neutrophilic 55KDa band is not SUMO-IκBα.

3.8. Expression of IκBα in 15°C treated neutrophils

As previously described by Pryde *et al.* (2000), isolated neutrophils treated at 15°C for 20 hours undergo substantial low percentage of apoptosis, while constitutive apoptosis may reach nearly 80% when cells were kept at 37°C. After the treatment of 15°C, when the cells were subsequent warming up to 37°C , rapid apoptosis was observed (e.g., 100% apoptosis at 120 minutes, while 15°C treated cells resist at about 10% apoptosis). Cells treated between 10 and 20°C has been shown for the



Anti-SUMO

Figure 3-7; 55KDa IκBα is not SUMO- IκBα complex in neutrophil cytoplasm. Purified neutrophils (5.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum for 30 minutes. Using anti-SUMO antibodies, 55KDa IκBα is denied to be SUMO conjugated IκBα while in Jurkat cells (Jurk) 55KDa is shown as a SUMO positive band, possibly SUMO-IκBα.

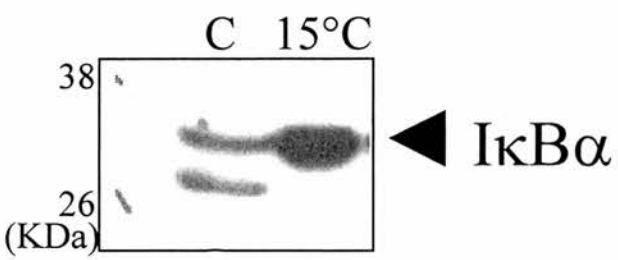


Figure 3-8; Expression of IκBα from 15°C treated neutrophils. Purified neutrophils (5.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum for 30 minutes, indicated as 'C' or cultured at 15°C for 20 hours (Pryde *et al.*, 2000). In 15°C treated cells, IκBα is expressed as only one band at 37KDa. In contrast, neutrophils incubated for 30 minutes in the medium express three bands of IκBα (but only two are shown). Anti-IκBα antibody, 10B, was used.

lower metabolic activities, such as reduction in membrane lipid fluidity, a decrease in the rate of protein translation, and an inhibition of vesicular tracking and neutrophil respiratory burst activities (Liu *et al.*, 1996). Pryde *et al.*, (2000) has shown the inhibition of pro-caspase 3 in the cells which has been treated at 15°C but reversed this inhibition to the active state when cells were warming up to 37°C. Also, this action was reversed with the addition of zVAD, a protease inhibitor, suggesting the crucial role of caspase pathway for neutrophil apoptosis in temperature dependent manner.

Neutrophils were incubated for 15°C for 20 hours (Pryde *et al.*, 2000) resulting in their apoptosis being arrested. These cells were harvested and subjected to WB analysis for expression of IκBα. Figure 3-8 demonstrates that 15°C treated neutrophils express only one band, 37KDa of IκBα, which was recognised by 10B. The explanation of different IκBα expression between one band and three bands in the different treatments is unknown. However, this may suggest lower cellular metabolism (Liu *et al.*, 1996) or arrest of caspase pathways may be involved with cleavage of IκBα (30k Da) or conjugation of some proteins onto IκBα (55k Da). (See discussion 3.16. for more details.)

Summary

- It is possible to detect only one band of ordinary IκBα, 37KDa, in neutrophils if cells were incubated at 15°C for 20 hours to arrest spontaneous apoptosis.

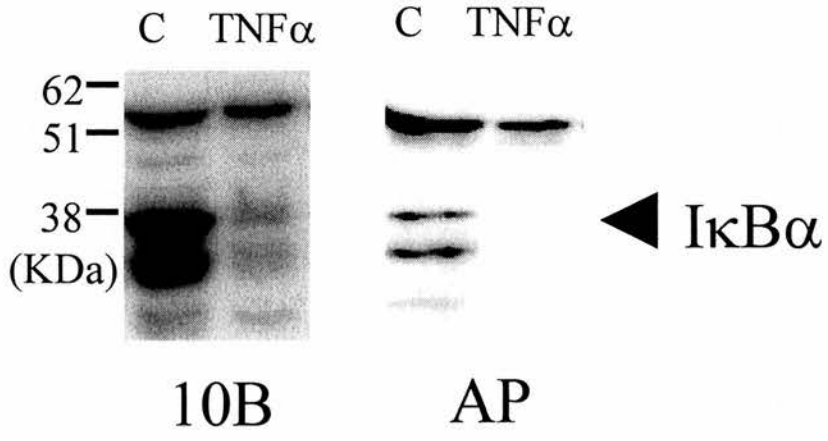


Figure 3-9; Expression and degradation of IκBα by TNFα stimulation. Purified neutrophils (5.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum with or without TNFα (10ng/ml) for 30 minutes. Cells were lysed for cytoplasm extract and being examined by WB. Different anti-IκBα antibodies, 10B and AP are used to determine the expression. Both antibodies detect three bands of IκBα. Upon TNFα stimulation, the two bands (37K and 30KDa) were degraded.

3.9. Degradation of IκBα by TNFα

When TNFα stimulates cells, IκBα is phosphorylated and this results in the degradation of IκBα by the proteasome. As demonstrated in figure 3-9, IκBα degradation upon TNFα (10ng/ml) stimulation occurs in neutrophils. They are detected by anti-IκBα antibodies 10B and AP. The two bands, 30K and 37KDa are degraded while 55KDa band remains in the cytoplasm. Normally IκBα is expressed as 37KDa, thus, degradation of 37KDa of IκBα is expected. However, the cause of degradation of 30KDa of IκBα upon TNFα stimulation is unknown.

Summary

- Using different antibodies, 37KDa and 30KDa bands of IκBα are detected to be degraded upon TNFα stimulation.
- The 55KDa band of IκBα remains in the cytoplasm upon TNFα stimulation.

3.10. IκBα in neutrophils

To assess the function of IκBα in neutrophils, neutrophils were cultured with different stimuli, such as TNFα (10ng/ml), LPS (500ng/ml), gliotoxin (100ng/ml), Mg132 (20μM) and caspase-3 inhibitors (50μM). The results are shown in figure 3-10. Anti-IκBα antibody, 10B, was used in this experiment. In control condition (C) medium containing 10% autologous serum (M+S) showed better signals (stronger signals) compared to PBS or medium only. All other conditions contain 10% autologous serum. In all non-treated control conditions (PBS, M, M+S), three bands of IκBα were detected.

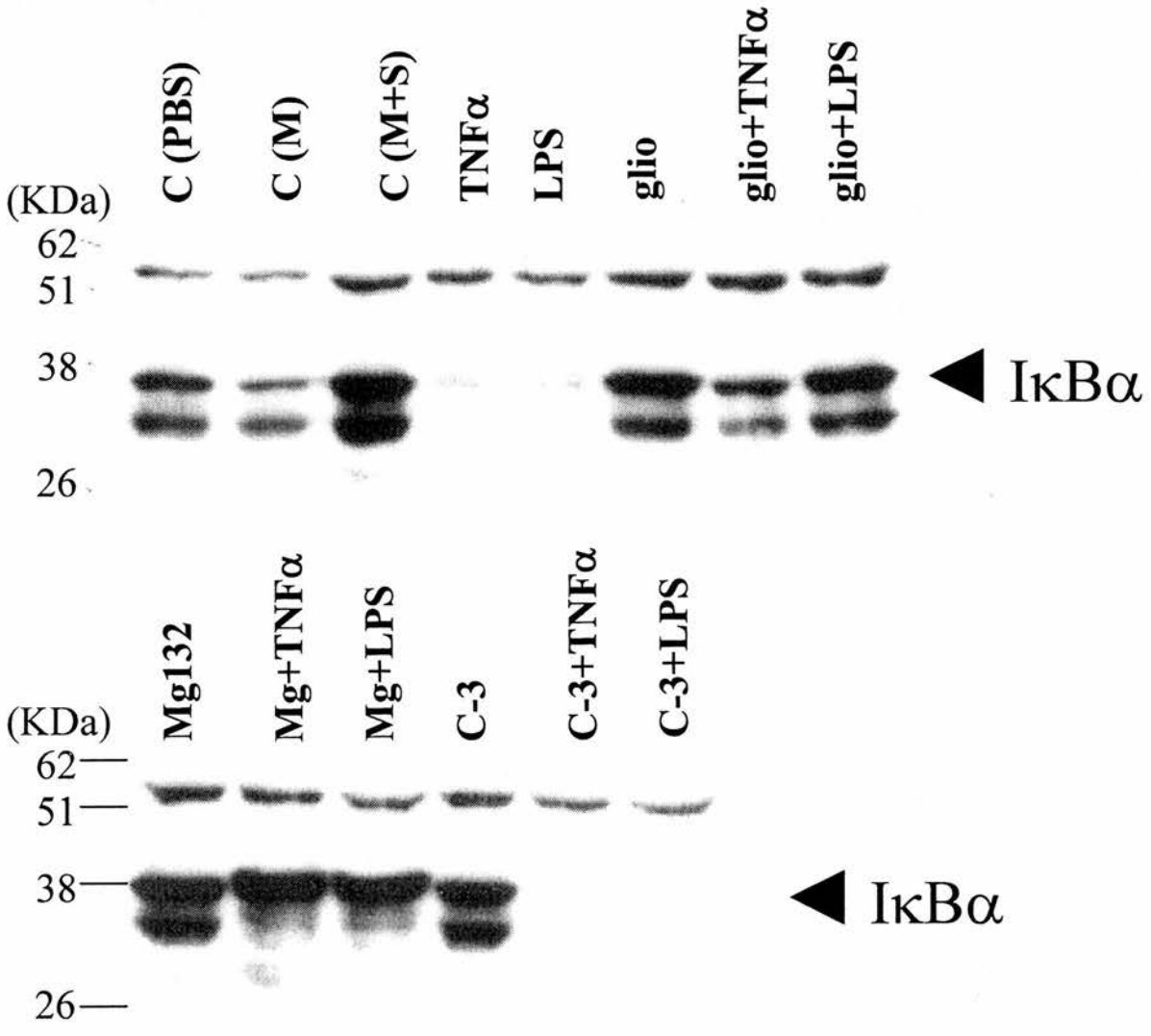


Figure 3-10; Expression and degradation of IκBα. Purified neutrophils (5.0×10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum were treated with different stimuli for 30 minutes before TNFα (10ng/ml) and LPS (500ng/ml) were added for a further 30 minutes. 'C' indicates control condition without any stimulation. 'M' and 'S' indicate medium and serum respectively. Cells were lysed for cytoplasmic extract and examined by WB, with using anti-IκBα antibody, 10B. TNFα and LPS degrades two bands of IκBα, while gliotoxin (glio) prevents those degradations and proteasome inhibitor, Mg132 (Mg) (20μM), prevents only one band degradation. Caspase-3 inhibitor (C-3) (50μM) did not affect the expression of IκBα.

Upon TNFα and LPS stimulation, two bands of IκBα, 37K and 30KDa, were degraded. The degradation of 37KDa IκBα leads to the activation of NF-κB. These results correlate with our previous work (Ward *et al.*, 1999, see appendix) where induction of NF-κB activation by gel shift assay stimulated with TNFα and LPS was shown. 30KDa IκBα also is able to be degraded by stimulation with TNFα and LPS, but the mechanism and function of its degradation is unknown.

In this experiment, gliotoxin and proteasome inhibitor Mg132 were used as NF-κB inhibitors. These NF-κB inhibitors were preincubated with cells for 30 minutes before TNFα or LPS were added. Then after a further 30 minutes, cells were incubated with TNFα or LPS in the indicated conditions.

As described in 4.1.4. in chapter 4, gliotoxin is an epipolythiodioxoperazine produced by the fungus *Aspegillus fumigatus*, which exhibits immune suppressive activity both *in vitro* and *in vivo* and selectively inhibits NF-κB activation (Pahl *et al.*, 1996). We (Ward *et al.*, 1999) have also shown that gliotoxin inhibits NF-κB in neutrophils and induces apoptosis, which is enhanced by co-culture with TNFα.

Figure 3-10 demonstrates that gliotoxin prevented IκBα degradation by itself or with TNFα or LPS stimulation. Although TNFα and LPS showed degradation of 37K and 30KDa of IκBα, gliotoxin prevents TNFα - or LPS- induced degradation. These results indicate the inhibition of NF-κB, which was also showed previously from our results (Ward *et al.*, 1999).

Similarly, the proteasome inhibitor Mg132 inhibited the degradation of 37KDa IκBα. However, interestingly, the 30KDa IκBα was not prevented from degradation on co-culture with TNFα and LPS. This may indicate that 30KDa IκBα may be degraded by a different mechanism but not by 26S proteasome.

On the other hand, caspase-3 inhibitors did not prevent TNFα- or LPS- induced degradation of 37K and 30KDa IκBα.

Although 37K and 30KDa of IκBα were degraded by stimulation with TNFα or LPS, the 55KDa band of IκBα still remains unaffected.

Summary

- In unstimulated resting state, three bands (55K, 37K and 30KDa) of IκBα were recognised.
- Both 37K and 30KDa IκBα were degraded under TNFα or LPS stimulation, suggesting activation of NF-κB.
- Gliotoxin inhibited TNFα- or LPS- induced degradation of 37K and 30KDa of IκBα.
- Mg132 inhibited the degradation of 37KDa IκBα only when TNFα and LPS were added.
- 37KDa IκBα was degraded by the 26S proteasome.
- 30KDa IκBα may not be degraded by the 26S proteasome.
- 30KDa IκBα may be degraded by some mechanism that gliotoxin can inhibit.

- Caspase-3 inhibitors did not show any influence on IκBα degradation upon stimulation with TNFα and LPS.

3.11. Neutrophil apoptosis induced by various NF-κB inhibitors

To investigate the effect of various NF-κB inhibitors on constitutive neutrophil apoptosis, neutrophils were exposed to Mg132 (20μM), PDTC (300μM) and curcumin (20μM) for up to 20 hours. All these drugs has been shown to inhibit NF-κB, (Moynagh *et al.*, 1994, Singh *et al.*, 1995, Arenzana-Seisdedos *et al.*, 1995) therefore, these drugs have been tested on constitutive neutophil apoptosis. The results are shown in figure 3-11 (see appendix for neutrophhil apoptosis induced by gliotoxin).

*The usage of other NF-κB inhibitors were essential since gliotoxin which may influence other pathways. It is necessary to use various types of inhibitors because some might be non-specific to the certain intracellular pathway. This is the reason why we had developed TAT-IκBα as a specific tool to investigate NF-κB in eosinophils (see chapter 5 for more details).

The proteasome inhibitor, Mg132 prevents degradation of IκBα (37KDa and 30KDa) (figure 3-10), and induce apoptosis (figure 3-11). At 20 hours Mg132 induces to up to 100% neutrophil apoptosis whereas control condition induces about 65%. This suggests that inhibition of NF-κB may be the one of the direct causes of induction of apoptosis in neutrophils.

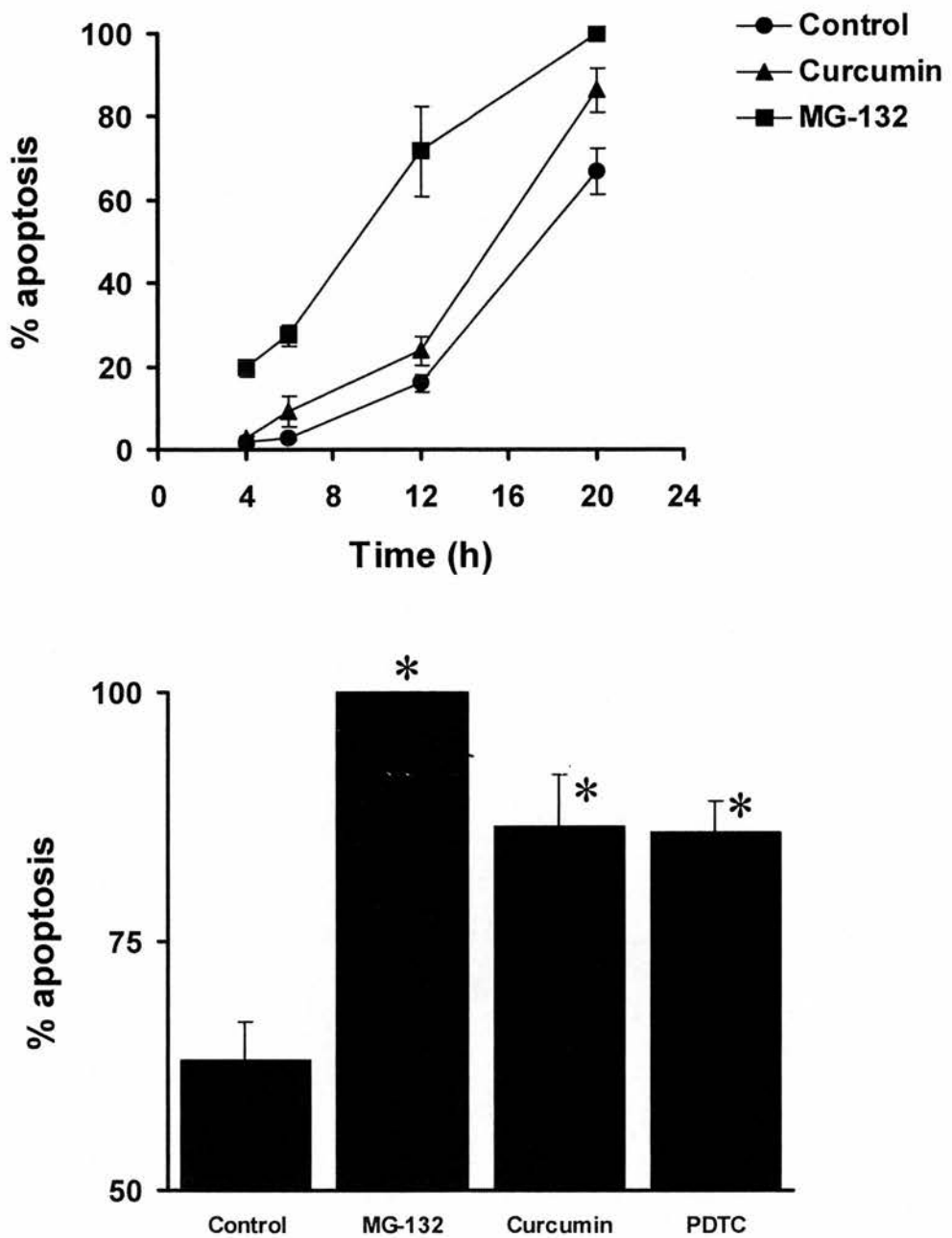


Figure 3-11; Effect of various NF-κB inhibitors on neutrophil apoptosis. Purified neutrophils (5.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum, were exposed to various NF-κB inhibitors, Mg132 (20μM), curcumin (20μM) and PDTC (300μM) for 20 hours. After incubation, cells were harvested and assessed morphologically for apoptosis. All values represent mean ± S.E. of n=6 experiments, each perfomed in triplicate ($P<0.02$). * represents significance ($p<0.05$) from control.

As other NF-κB inhibitors, curcumin, which is known as anti-inflammatory reagents and suppression of NF-κB (Jobin *et al.*, 1999) and PDTC (Moynagh *et al.*, 1994) (examined only at 20 hours time point in figure 3-11) were used as to investigate their effect on induction of neutrophil apoptosis (figure 3-11). Both curcumin and PDTC exerted their effect to induce apoptosis, although limited (especially curcumin at 12 hours, there is small difference but statistically significant from the control) compared to Mg132 or gliotoxin.

For statistical analysis please refer 2.13.

Summary

- Mg132 itself induces neutrophil apoptosis.
- Curcumin and PDTC induce neutrophil apoptosis.

3.12. The existence of NF-κB (p50 and p65) in eosinophils.

Unlike using cell lines, to study eosinophil also involves some technical difficulties, such as purified cell numbers, proteases and the eosinophil's short life. Despite these technical difficulties, eosinophils were studied by WB and immunofluorescence staining in this thesis. These studies show for the first time the role and function of NF-κB in eosinophils. In this chapter, expression of p50, p65, IκBα and IκBβ in eosinophils are shown. Then in the following chapters the role of NF-κB in eosinophils are investigated.

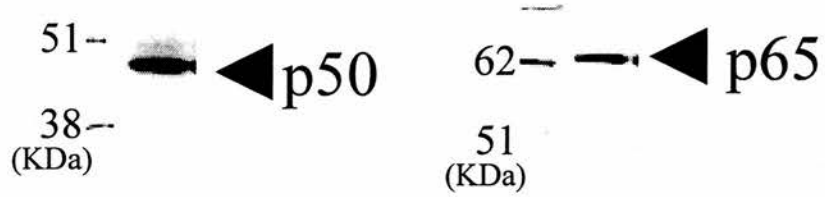


Figure 3-12; Expression of p50 and p65 in eosinophils. Purified eosinophils (3.0 x 10^6 cells/ ml) were lysed for cytoplasmic extract and examined by WB. Anti-p50 and p65 antibodies were used to determine the expression.

To investigate whether eosinophils possess any dimers of NF-κB, cytoplasmic lysate was examined by WB, probing with p50 and p65 antibodies. p50 and p65 are predominantly common heterodimers of NF-κB. The results are shown in figure 3-12. As for the first time by WB it is demonstrated that eosinophils possess both p50 and p65. In this thesis, p65 was examined as one of the dimers of NF-κB by WB and by immunofluorescence microscopy (see chapter 4 and 5).

Summary

- Eosinophils do possess both p50 and p65.

3.13. IκBα degradation by TNFα in eosinophils

To investigate the expression of IκBα in eosinophils, eosinophil cytoplasmic extract was examined by WB and probed with anti-IκBα antibody. As shown in figure 3-13, for the first time, IκBα expression was shown in the cytoplasm of eosinophils. Unlike neutrophils, IκBα in eosinophils is indeed expressed only as 37KDa.

IκBα is expressed in the cytoplasm in the resting state (0 min). Upon TNFα stimulation for various time points, IκBα is degraded. Thus, these results indicate that NF-κB activation in eosinophils stimulated with TNFα.

Summary

- Eosinophils possess IκBα in the cytoplasm.
- IκBα is expressed as a 37KDa.
- IκBα in eosinophils is degraded by TNFα stimulation.

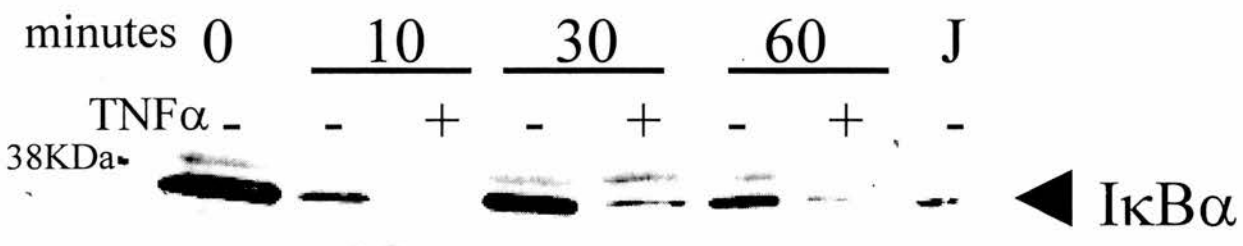


Figure 3-13; Expression and degradation of IκBα by TNFα stimulation. Purified eosinophils (3.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with TNFα (10ng/ml) for the time indicated. 'J' indicates cytoplasmic extract from Jurkat cells. Cells were lysed for cytoplasmic extract and examined by WB. Only one band of IκBα (37KDa) is expressed and degraded by TNFα stimulation.

3.14. IκBβ in eosinophils

The common isoforms of the family of IκB in mammalian cells are IκBα, IκBβ, and IκBε, which all contain ankyrin repeats and hold NF-κB in the cytoplasm. IκBα is the most abundant isoform of IκB. On the other hand, IκBβ is a relatively minor form of IκB and also believed to undergo signal-induced phosphorylation, ubiquitination, and degradation (DiDonato *et al.*, 1996). However, the kinetics of IκBβ degradation is significantly slower than IκBα (Thompson *et al.*, 1995), indicating the lower efficiency of ubiquitination and phosphorylation than IκBα.

In figure 3-14, for the first time, IκBβ (45KDa) expression in eosinophils is shown. Since the mechanism of IκBβ degradation is not known especially in eosinophils, in this experiment a time point of 30 minutes was chosen for investigation. There was no degradation or other specific changes observed at 30 minutes, which may not be the most suitable time point for any changes. However, as IκBβ is thought to be the minor form of IκB further experiments were not performed.

Summary

- IκBβ is expressed in eosinophil cytoplasm.

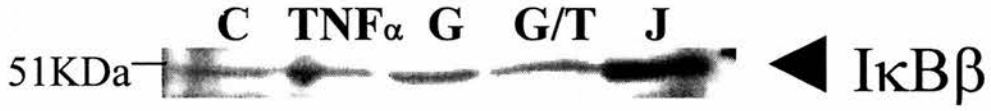


Figure 3-14; Expression of IκBβ. Purified eosinophils (3.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with TNFα (10ng/ml), gliotoxin (G) (100ng/ml) or gliotoxin and TNFα (G/T) for 30 minutes each. 'J' indicates cytoplasm extract from Jurkat cells. Cells were lysed for cytoplasmic extract and examined by WB.

3.16. Discussion

Neutrophils and eosinophils are derived from the same myeloid precursor yet are different in their nature and metabolism for cellular activities. In this chapter the work presented on the neutrophil is a part of the study from our previous publication (Ward *et al.*, 1999) to investigate further expression of IκBα which is an index of measuring NF-κB activation. On the other hand, in eosinophils, for the first time the expression of p50, p65, IκBα and IκBβ were shown. In the following chapters (4, 5 and 6), the role of NF-κB in eosinophil survival and apoptosis pathways were investigated.

Neutrophil experimental conditions

Neutrophils have been known to be one of the most difficult cells for biochemical studies due to their high content of proteases which can disturb the target protein during its preparation. Therefore, inhibition of proteases is one of the key targets for experimental methods. As shown, various antibodies and conditions express different signals. Therefore, various conditions of the neutrophil culture have been attempted to obtain consistent results. For example, for unknown reasons, when neutrophils were cultured with medium containing serum, the signals on WB analysis was enhanced compared to the ones without. Autologous serum contains various substances including anti-apoptotic agents which may prevent protease activation. This may have influenced the preparation of lysate; e.g., protease activities may have inhibited as the result in the better signals. This reason might also occur with when cells were incubated at 15°C for 20 hours. Both conditions exert as anti-apoptosis, therefore, inhibition of protease activity might stabilise the intracellular protein, resulting in better expression on WB. However, to overcome

protease problems, as McDonald and Cassatella (1998) suggested, nitrogen cavitation and the usage of DFP might be essential for isolation of intracellular protein from neutrophils. However, to set up those methods in our laboratory were concluded to be beyond practical usage and not suitable despite the fact that they might be the best option for neutrophil experiments.

IκBα in neutrophils

As it has been shown, expression of IκBα in neutrophils showed three distinct bands 55K, 37K and 30KDa, which are not seen in the other types of cells. For example in eosinophils, only 37KDa of IκBα is expressed. The 55KDa band was demonstrated not to be SUMO-conjugated IκBα. In addition, the stimulation with TNFα or LPS, 55KDa band is undegradable. If this is a real IκBα band, some conjugated protein may protect from TNFα- or LPS-induced degradation. In that case it could be that 55KDa IκBα is a super suppressive form of IκBα. Interestingly, this band (as well as 30KDa band) was never observed in the cells cultured at 15°C for 20 hours. Therefore, it could be due to some apoptotic pathway involved with protein conjugated with IκBα. Therefore, the 55KDa band was denied to be a non-specific band such as IgG, which is expressed around 55K-60KDa, because of the lack of expression in 15°C treated cells.

On the other hand, the 37KDa band of IκBα was expressed and degraded as be expected. It was degraded by TNFα and LPS stimulation and prevented by gliotoxin and Mg132. The exact mechanism of inhibition of NF-κB by gliotoxin is currently unknown (see chapter 4 discussion for the hypothesis of function of gliotoxin).

However, it may work through disruption of the IKK complex therefore, no phophorylation on IκBα occurs. Thus, this could be the reason for over-expression of IκBα in the cytoplasm.

The 30KDa band was also degraded by stimulation by TNFα and LPS. The degradation was prevented by gliotoxin but curiously not the proteasome inhibitor Mg132. This might suggest that this 30KDa band is degraded by a different mechanism distinct from the 26S proteasome.

These studies are extended from our previous studies (Ward *et al.*, 1999) and confirmed the inhibition of NF-κB by over-expression of IκBα in the cytoplasm by various NF-κB inhibitors.

Eosinophils

Biochemical experiments on eosinophils may not be as technically challenging as neutrophil works, therefore, the choice of antibodies, conditioned mediums, protease inhibitors are wider than used for neutrophil works. However, the difficulties of working with eosinophils are the cell number and the isolation methods. It was not guaranteed after the isolation each time that the desired number of the cells were harvested. In addition, the percentage of eosinophils from the granulocyte population is dependent on the donor. To obtain consistent results, the eosinophil works presented here in the following chapters have been taken mainly from 'high eosinophilic' donors.

These results show for the first time the existence of p50, p65, IκBα and IκBβ in the cytoplasm of eosinophils. Like other types of cells, eosinophils do possess NF-κB and its pathway, therefore, the role of NF-κB is investigated further in the following chapters.

Chapter 4
The inhibition of NF-κB un-masks the ability of TNFα to induce apoptosis in eosinophils.

4.1. Introduction

4.1.1. Eosinophil apoptosis

In inflammatory diseases such as asthma inappropriately recruited or activated eosinophilic granulocytes may cause damage to the tissue in the respiratory tract by release of their toxic contents (e.g., MBP, free O_2 radicals, etc.,) (see introduction 1.1.1. for eosinophilic toxic granules contents). Thus, eosinophil-mediated epithelial cell damage in the respiratory tract and surrounding tissue is likely to contribute to the pathogenesis and propagation of asthma and other inflammatory diseases such as allergic rhinitis.

Such inflammation may be resolved by efficient removal of activated eosinophils from inflammatory sites. Ligation of specific cell surface receptors and the consequent triggering of diverse signal transduction pathways control eosinophil functional responsiveness (Stern *et al*, 1992) and physiological programmed cell death or apoptosis (Stern *et al*, 1996). During apoptosis eosinophils undergo a series of characteristic changes (e.g. shrinkage, chromatin condensation, expression of cell surface phosphatidylserine) to shutdown their secretory capacity of toxic cell contents and marks them for 'silent' removal from inflamed sites by macrophages (Savill *et al.*, 1989) and other cells including epithelial cells (Walsh *et al.*, 1999).

Apoptosis is regulated by extracellular stimuli, such as TNFα, FasL, and their receptors by activating various death pathways such as the caspase pathway (see introduction 1.4.2. for more details). However, the pro-inflammatory cytokine TNFα can also trigger the NF-κB pathway for transcriptional activity which is responsible for synthesis of survival and pro-inflammatory proteins. There is a

delicate balance between these dual pathways, pro-survival and pro-apoptotic, generated by TNFα. Therefore, inhibition of TNFα -mediated NF-κB activation ultimately un-masks the caspase-dependent pro-apoptotic properties of TNFα (Beg and Baltimore, 1996, Wang *et al.*, 1996, van Antwerp *et al.*, 1996).

4.1.2. TNFα in granulocytes

TNFα is capable of priming neutrophils; rendering them more susceptible to activation upon subsequent exposure to other neutrophil secretagogues (O'Flaherty and Rossi, 1993, O'Flaherty *et al.*, 1991). Furthermore, TNFα can influence apoptosis in many cells including granulocytes (Ward *et al.*, 1999, Murray *et al.*, 1997, Temkin and Levi-Schaffer 2001); effects that may be controlled by activation of NF-κB (Ward *et al.*, 1999). For example, apoptosis is promoted when neutrophils are exposed to TNFα for short periods of culture (i.e., 2-8 hours) in a concentration-dependent manner. When apoptosis is assessed after 20 hours of TNFα exposure apoptosis is delayed (Murray *et al.*, 1997).

In human eosinophils TNFα has been shown to exert an anti-apoptotic effect that signals partly via the p38 MAP kinase pathway (Tsukahara *et al.*, 1999) and more recently Temkin and Levi-Schaffer (2001) showed that TNFα mediated eosinophil survival occurs via ligation of both TNF receptor subtypes and through the generation of the eosinophil survival factor GM-CSF; an effect that seems to involve activation of NF-κB.

4.1.3. NF-κB in eosinophils

We (Ward *et al.*, 1999) and others (Temkin and Levi-Schaffer 2001, McDonald *et al.*, 1997) have shown that NF-κB is present in neutrophils and in eosinophils (Fujihara *et al.*, 2001, Yamashita *et al.*, 1999), however role of NF-κB in the regulation of eosinophil derived chemokine production and apoptosis remains poorly understood.

Inflammatory cells have the capacity to synthesise and respond to NF-κB regulated pro-inflammatory cytokines. Eosinophils have the capacity to release inflammatory mediators derived from lipid metabolism and are capable of synthesising pro-inflammatory cytokines (e.g., IL-8, TNFα, GM-CSF, IL-5, etc.,) (reviewed by Giembycz and Lindsay, 2000) many of which are regulated totally or partially by NF-κB.

NF-κB is held in the cytoplasm by being bound to an inhibitory protein from the IκB family where 37KDa IκBα is the major form. Activation of NF-κB is mediated by signal-induced phosphorylation via the IKK complex by TNFα and degradation of its inhibitor, IκBα in the cytoplasm. Degradation of IκBα in the cytoplasm and translocation of NF-κB into the nucleus is induced by stimuli such as TNFα. (See figure 1-6-1 in introduction.) In this study to elucidate the role of NF-κB in eosinophils, the expression of IκBα in the cytoplasm and translocation of p65 was determined with the use of the NF-κB inhibitors, gliotoxin and Mg132.

4.1.4. Gliotoxin and Mg132 as NF-κB inhibitors

In this chapter, Mg132 and gliotoxin were used for inhibition of NF-κB.

Gliotoxin

Gliotoxin is produced by the fungi *Candida albicans* and *Aspergillus fumigatus*, and can cause several prominent biological effects, including immunosuppression. This fungal infections are sharply increasing and causes 40% of deaths in patients with acute leukaemia at some hospitals (Bodey *et al.*, 1992). Gliotoxin causes immunosuppression by disturbing the function and apoptosis of T cells, B cells (Müllbacher and Eichner, 1984), thymocytes and spleen cells (Sutton *et al.*, 1994), and macrophages (Waring *et al.*, 1988).

Gliotoxin belongs to the epipolythiodioxopiperazine class of secondary metabolites. *In vitro*, it has been shown that at relatively low concentrations, it exerts its effect as a specific NF-κB inhibitor (Pahl *et al.*, 1996) but at higher concentration, it disturbs the covalent interaction of disulphide formation in polypepties (reviewed by Waring and Beaver, 1996). Moreover, gliotoxin raises cyclic AMP and PKA activation and phosphorylation of histone H3 to induce apoptosis in thymocytes and certain cell lines (Waring *et al.*, 1997). The mechanism and function of gliotoxin to cause immunosuppression is not fully understood. However, the suppression of NF-κB activation resulting in inhibition of pro-inflammatory mediator formation is a likely explanation. Furthermore, inhibitor of NF-κB may be the mechanism for the apoptosis seen in some cells following gliotoxin treatment.

Mg132

On the other hand, upon phosphorylation of IκBα, 26S proteasome degrades IκBα for releasing of NF-κB into nucleus for the trascriptional activation. Proteasome inhibitors are broadly used for the study of NF-κB (Traenchkner *et al.*, 1994). When proteasome inhibitors are applied to cells, no degradation of IκBα occurs and results in the accumulation of IκBα in the cytoplasm thereby retaining NF-κB in the cytoplasm. In this thesis, specific proteasome inhibitor Mg132 (z-Leu-Leu-Leu-CHO) (Bush *et al*, 1997) was used.

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In this chapter, the role of NF-κB in eosinophils was investigated using gliotoxin and Mg132 as inhibitors of pathway.

<u>The aim of this chapter</u>

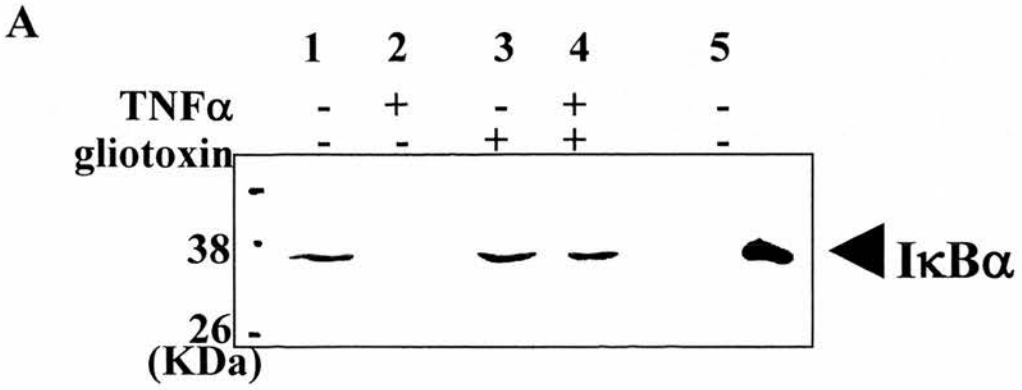
- To investigate the role of NF-κB in eosinophil apoptosis.
- To investigate if inhibition of NF-κB induces eosinophil apoptosis.
- To investigate the expression of IκBα in the cytoplasm in eosinophils.
- To investigate the translocation pattern of p65 in eosinophils.
- To investigate NF-κB-dependent IL-8 synthesis.

4.2. - 4.5. Results

4.2. TNFα induced IκBα degradation in the cytoplasm in eosinophils

Upon appropriate cell stimulation, IκBα is rapidly phosphorylated via the IKK complex and undergoes degradation by the 26S proteasome thereby permitting NF-κB to translocate from the cytoplasm to the nucleus. Since phosphorylation and subsequent degradation of IκBα is a prerequisite for NF-κB activation, the amount of IκBα in cytosolic extracts of eosinophils was examined by Western blotting (WB) analysis as an index of the NF-κB activation. NF-κB inhibitors, gliotoxin (100ng/ml) and Mg132 (20μM) were incubated with eosinophils for 30 min prior to the addition of TNFα (10ng/ml) for a further 30 min. Then cells were harvested for the following experiments.

In quiescent un-stimulated cells, IκBα appeared as a single band of 37KDa, that disappeared following TNFα stimulation indicating degradation of IκBα as shown in figure 4-2-1 panel A. This TNFα-induced IκBα degradation was prevented by gliotoxin (lane 4). Furthermore, panel B shows the expression of p65 in the cytoplasm. The absence of p65 in the cytoplasm following TNFα stimulation (lane 2), is due to the translocation of p65 into the nucleus; an effect reversed by gliotoxin. Figure 4-2-2 demonstrates the evidence of translocation of p65 into the nucleus by immunofluorescent confocal microscopy analysis. Anti-p65 antibody conjugated with FITC (green) is observed in the cytoplasm in control conditions. Nuclei are stained with propidium iodide (red). Upon TNFα stimulation, p65 is translocated to the nucleus resulting in the formation of a yellow colour due to overlapping of FITC (green) and propidium iodide (red). Similar results are seen in



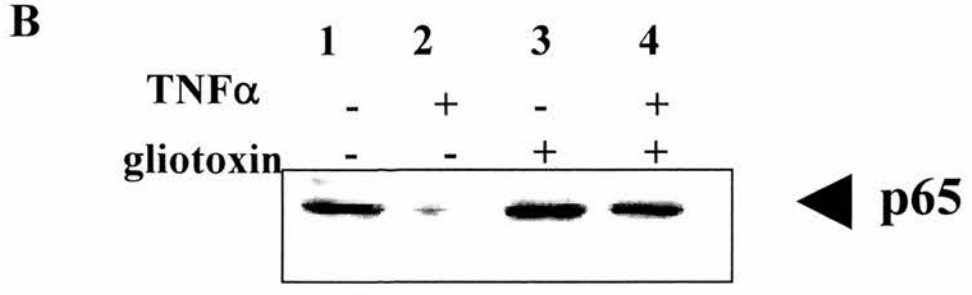


Figure 4-2-1; Western blot analysis of cytoplasmic IκBα and p65. Eosinophils were stimulated with TNFα (10ng/ml), gliotoxin (100ng/ml) for 15 min in Iscove's DMEM at 37°C and cytoplasmic extract prepared and analyzed by western blotting with anti-IκBα antibody, and anti-p65 antibody. Panel A shows cytoplasmic IκBα expression. Upon TNFα stimulation the IκBα band disappears indicative of proteosomal degradation, treatment of the cells with gliotoxin stabilizes IκBα in the cytoplasm both in the absence and presence of TNFα. For a positive control Jurkat cell cytoplasmic extracts were included (Lane 5). Panel B, shows the amount cytoplasmic p65. The loss of p65 expression in the cytoplasm with TNFα stimulation correlated with IκBα loss and suggests translocation of p65 into nucleus, a process blocked by gliotoxin. The blots are representative of at least 3 separate experiments.

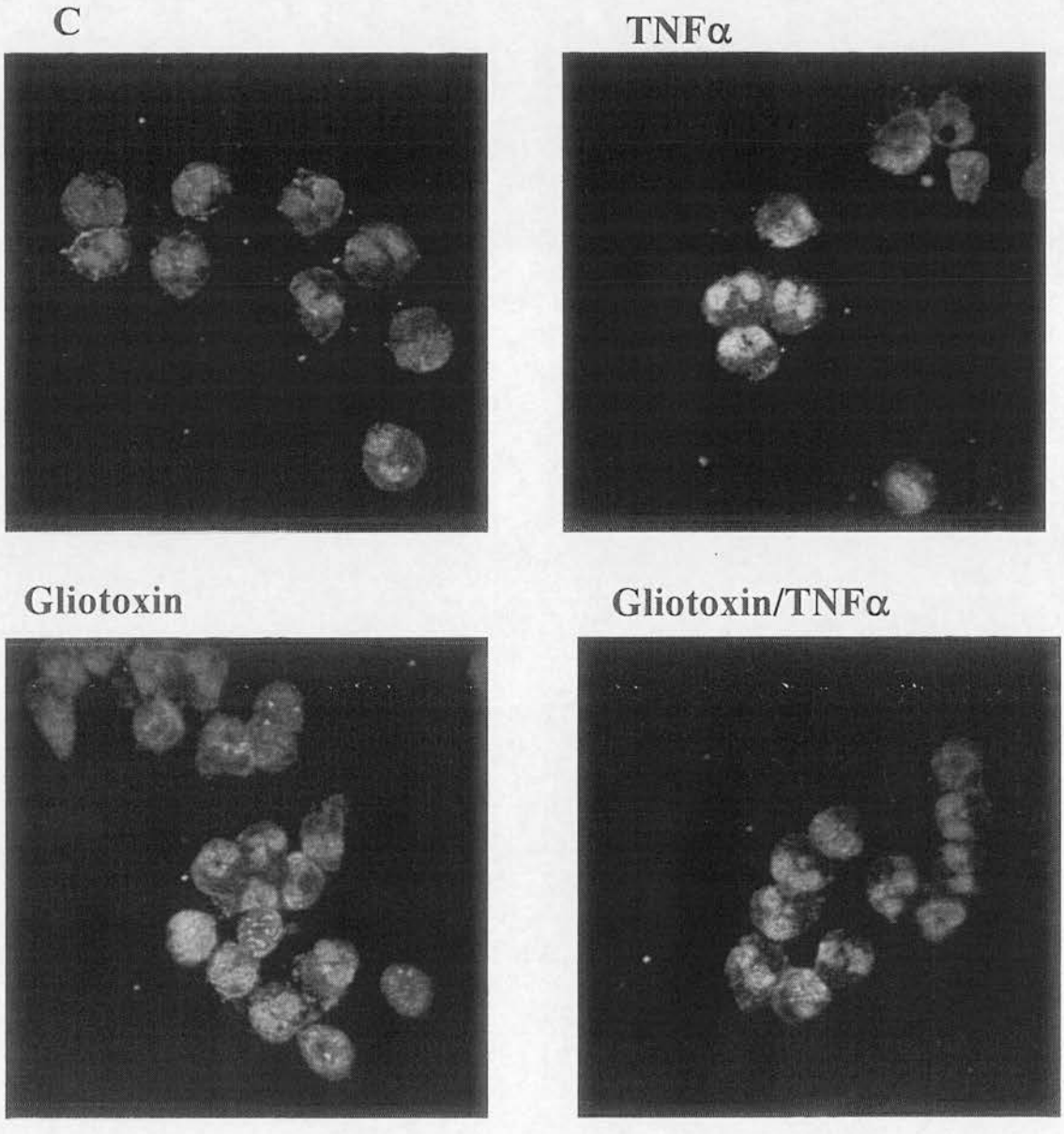


Figure 4-2-2; Confocal microscope images of p65 expression in eosinophils stimulated with TNFα with and without gliotoxin. Eosinophils were stimulated for 45 min in Iscove's with TNFα (10ng/ml) and gliotoxin (100ng/ml). Anti-p65 antibody was FITC labeled (shown as green) and the nucleus was stained with propidium iodine (shown as red). Upon TNFα stimulation p65 translocate into the nucleus, resulting in an enhanced yellow color. Gliotoxin, with or without TNFα, dramatically inhibits p65 nuclear translocation.

4-2-1 WB analysis, which showed IκBα degradation and absence of p65 in the cytoplasm. Similarly, gliotoxin reversed the effect of TNFα. As shown in figure 4-2-2, addition of gliotoxin prevented TNFα-induced translocation of p65, which correlates with the results of figure 4-2-1 which shows the over-expression of IκBα and presence of p65 in the cytoplasm.

Summary

- IκBα is degraded upon TNFα stimulation.
- Gliotoxin prevents TNFα-induced IκBα degradation.
- p65 is absent under TNFα stimulation in the cytoplasm.
- The absence of p65 in the cytoplasm by TNFα is prevented by gliotoxin.
- p65 translocates into nucleus following TNFα stimulation.
- Gliotoxin prevents TNFα-induced p65 translocation into nucleus.

4.3. Immunohistochemical analysis of NF-κB translocation in eosinophils

In order to provide compelling evidence for subcellular movement of p65 in eosinophils, quantitative immunohistochemical analysis of TNFα induced translocation of p65 was performed. Quantification of translocation is depicted in figure 4-3 A, B, and C where the immunofluorescent images are analyzed on a Leica Q550IW image analyser to detect p65 amount in the cytoplasm and nucleus and the results expressed as a ratio. In other words, area of green (anti-p65-antibodies-FITC) in the cells was measured by the image analyser, then, the area occupied by nuclei was detected by the presence of propidium iodide (red). The results in the area stained with green in nuclei or cytoplasm was calculated and shown as the ratio of p65 translocation into nucleus in figure 4-3. At least 1,000

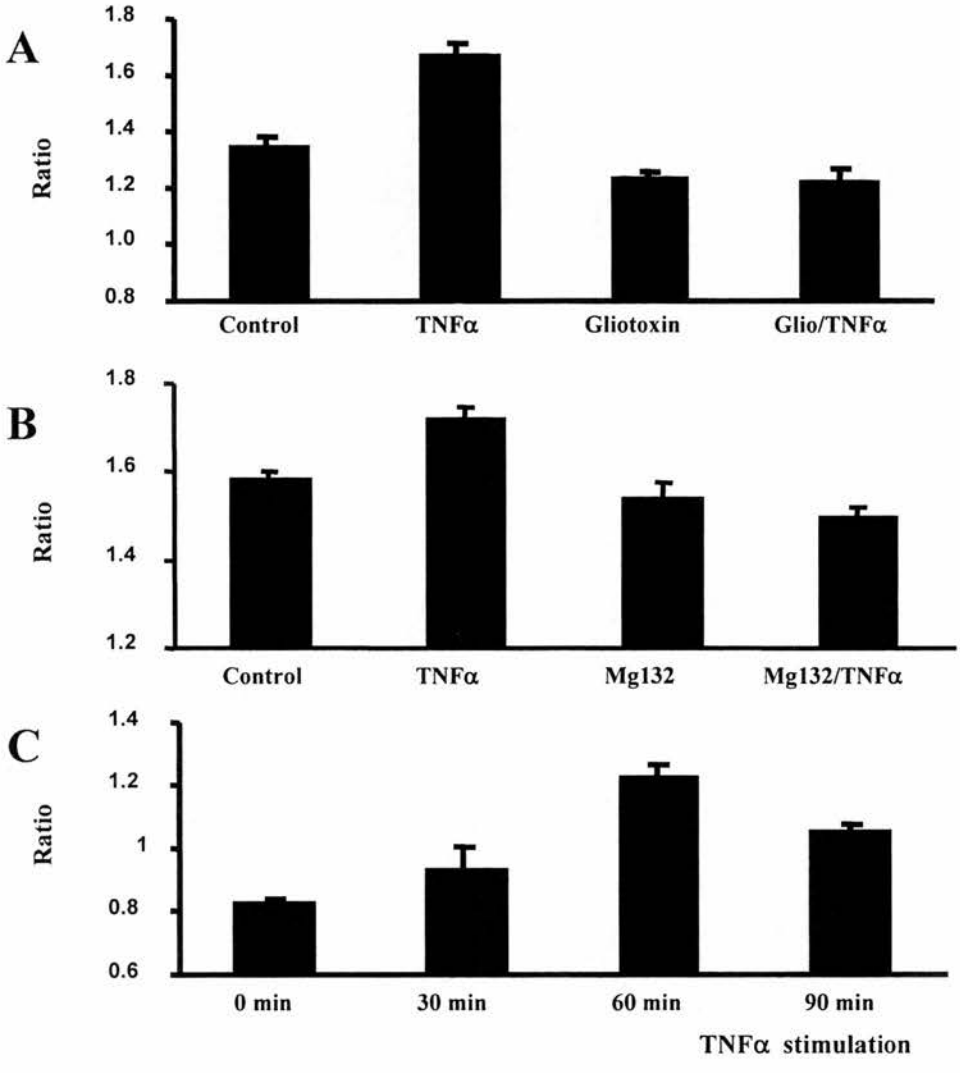


Figure 4-3; Quantitative analysis of p65 translocation to the nucleus. Computer analysis of p65 expression in the nucleus was measured as the ratio of p65-FITC nuclear fluorescence versus cytoplasmic fluorescence in each cell. At least 300 cells were counted per condition and the data is represented as mean ± SEM determined for each experiment independently. Eosinophils were incubated in Iscove's DMEM with the indicated stimuli (panels A and B) for 45 min and with TNFα for the indicated times (panel C). A, the effect of gliotoxin (100ng/ml) on activation by TNFα (10ng/ml). B, the effect of Mg132 (20μM) on p65 translocation into the nucleus. C, time-course of the effect of TNFα (10ng/ml). D. the effect of TNFα-induced translocation in each donor. The data are representative of one experiment of at least 3 similar experiments.

D

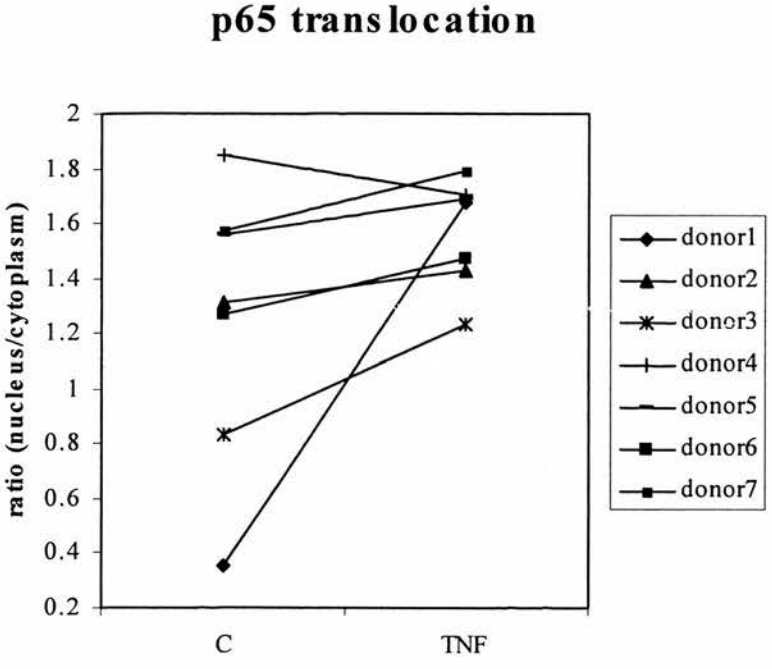


Figure 4-3-D; Quantitative analysis of p65 translocation to the nucleus

cells were assessed per condition. The standard error bar represents the error of the p65 translocation in each view which are typically 200 cells per view.

Figure 4-3 A and B clearly demonstrate the induction of translocation of p65 into the nucleus by TNFα. On the other hand, gliotoxin and Mg132 reverse TNFα-induced p65 translocation. Panel C demonstrates the time-course of translocation of p65 stimulated with TNFα. Maximum induction of TNFα stimulated p65 translocation in eosinophils isolated from this particular donor occurred at after 60 min exposure to TNFα. Following this time point, NF-κB levels in the nucleus gradually decrease, perhaps reflecting rapid re-synthesis of IκBα and consequent removal of NF-κB from the nucleus (Arenzana-Seisdedos *et al.*, 1995). Although different levels of basal and stimulated p65 distribution were observed between individual donors, substantial p65 translocation by TNFα stimulation was observed (figure 4-3-D). The response of the rate of p65 translocation stimulated by TNFα was donor dependent. However, the majority of donors had p65 translocation stimulated by TNFα except one donor (donor 4). Donor 4 recorded symptom scores as 'severe allergic rhinitis' on that day when the blood was taken. This could explain why basal activation of p65 was so high to compare with others but the stimulation with TNFα did not have any further activation of NF-κB.

Summary

- p65 translocation into nucleus occurs upon TNFα stimulation.
- TNFα-induced translocation of p65 is prevented by gliotoxin and Mg132.
- Maximum induction of TNFα-induced translocation of p65 occurred in this particular donor at 60 min.

- Different donors showed different rates of p65 translocation into nucleus stimulated by TNFα.

4.4. NF-κB activation mediates IL-8 production by human eosinophils.

It is established in many cell types (McKay and Cidlowski, 1999, Roebuck 1999) that IL-8 production is tightly controlled by NF-κB and eosinophils have the capacity to synthesise (Yamashita *et al.*, 1999). In other words, measuring IL-8 is indirect way to determine NF-κB activation. Because IL-8 is the important pro-inflammatory cytokine of the family of CXC chemokines, that strongly attracts and activates neutrophils, T lymphocytes. But the effect of IL-8 on eosinophils themselves may be still controversial (Petering *et al.*, 1999) although eosinophils express CXCR1 and CXCR2 (reviewed by Nagase *et al.*, 2001). However, IL-8 is a strong pro-inflammatory cytokines, and upregulated by NF-κB, the determination of IL-8 was thought to be appropriate for this project. The production of IL-8, which is another indicator of NF-κB activation, was measured by ELISA.

Figure 4-4 demonstrates that TNFα is a powerful stimulator of IL-8 production in eosinophils even at the early time point of 90 min, a time point chosen since there is no gliotoxin-induced apoptosis that could influence IL-8 production (data not shown). The production of IL-8 was dramatically suppressed by gliotoxin at a concentration that effectively inhibits NF-κB activation at this time point. This data correlates with figure 4-2-1 and 4-2-2 which demonstrate overexpression of IκBα in the cytoplasm and p65 translocation into the nucleus. Although in figure 4-2-1 the overexpression of IκBα in the cytoplasm is seen, it is likely that basal level of NF-

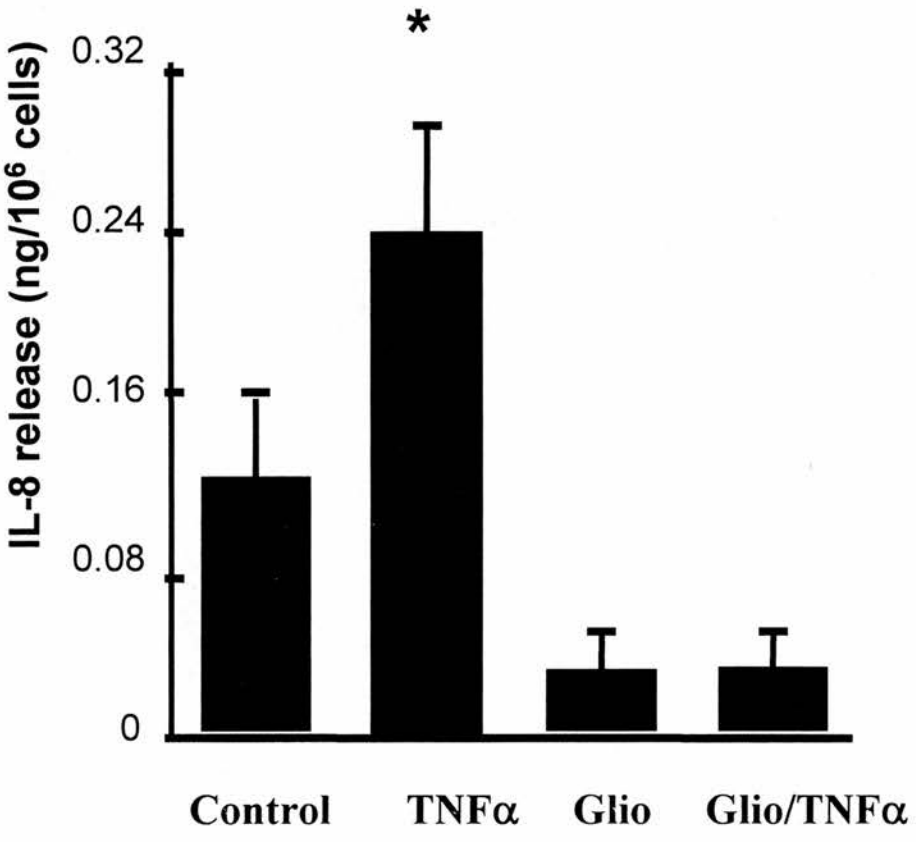


Figure 4-4; Effect of gliotoxin on TNFα induced IL-8 release from eosinophils. Eosinophils were stimulated with TNFα (10 ng/ml) for 90 min in the absence and presence of gliotoxin (100ng/ml) and the IL-8 concentrations in the cell supernatants determined by specific ELISA. The data are expressed as the mean ± SEM of 4 separate experiments using eosinophils isolated from different donors. Significant difference ($p<0.05$) from the control is indicated by *.

κB activation occurs during the resting state. This is why although un-stimulated control condition produce some of IL-8. On the other hand, stimulation with gliotoxin may suppress the basal level of NF-κB activation. Therefore, the more suppression of IL-8 production by gliotoxin is shown. There is no significant apoptosis at this time point.

Summary

- TNFα induces IL-8 production.
- TNFα-induced IL-8 production is prevented by gliotoxin.
- Gliotoxin may suppress the basal level of NF-κB activation.

4.5. NF-κB regulates eosinophil apoptosis

It has been shown that the TNFα-induced activation of NF-κB is inhibited by gliotoxin and Mg132. To investigate if NF-κB inhibition could induce apoptosis and un-mask the pro-apoptotic ability of TNFα the following experiments were performed. Figure 4-5-A demonstrates the effect of gliotoxin and Mg132 on TNFα–induced apoptosis. TNFα alone and gliotoxin alone cultured with eosinophils for 4 hours caused little morphological apoptosis, however when eosinophils were cultured in the presence of TNFα plus gliotoxin there was a dramatic induction of characteristic pyknotic nuclei typical of eosinophil apoptosis. Our preliminary data indicated that it requires a significant amount of time (> 3 hours) to observe significant apoptosis and that the 4 hours time point is optimal to

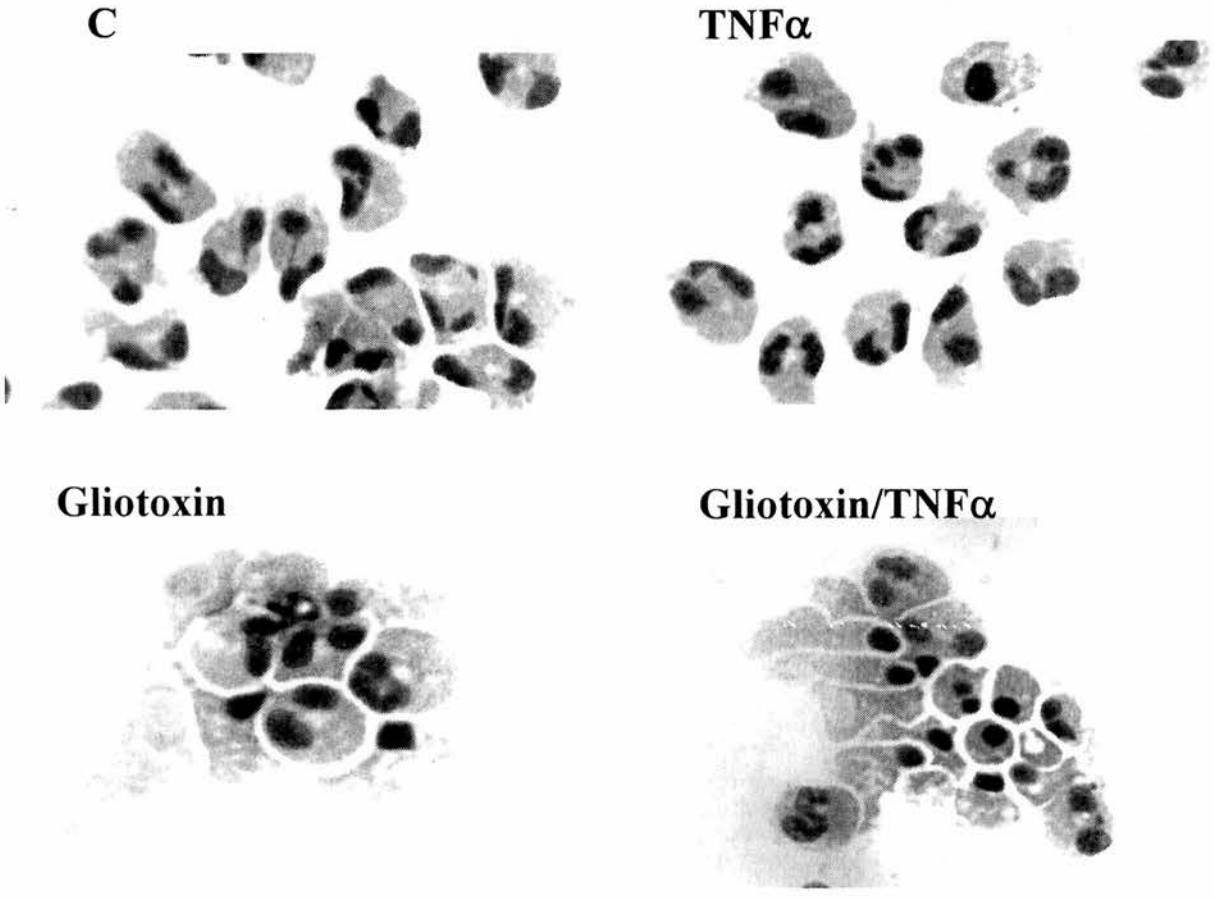


Figure 4-5-A; The effect of gliotoxin and TNFα on eosinophil apoptosis. Cytocentrifuge preparations were made of eosinophils incubated for 4 hours at 37°C in medium alone (control), TNFα (10ng/ml), gliotoxin (100ng/ml), and combination of these reagents. The rates of apoptosis in this experiment are control 5%, TNFα, gliotoxin 26.5% and gliotoxin/TNFα 47.5%.

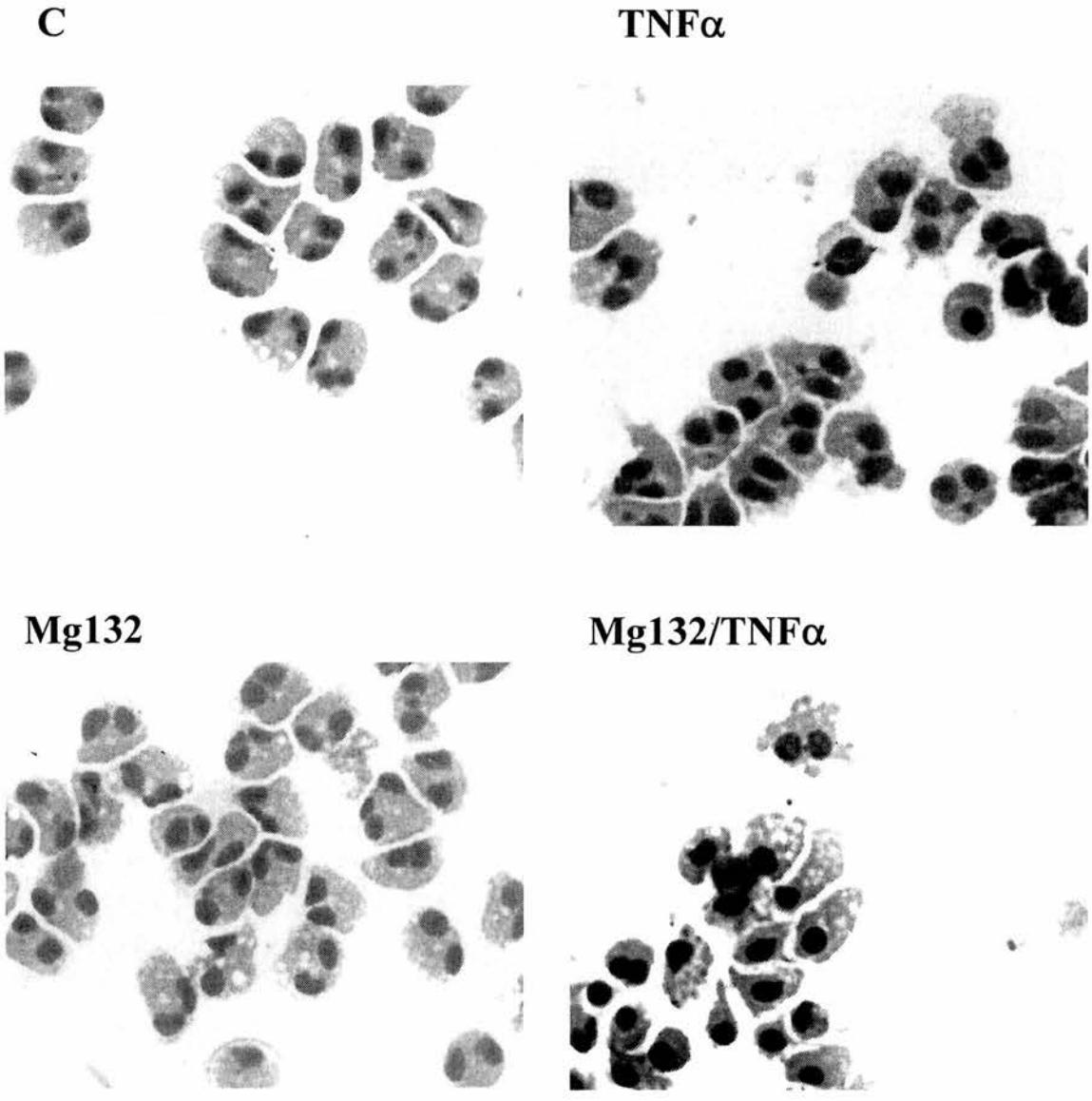


Figure 4-5-B; The effect of Mg132 and TNFα on eosinophil apoptosis. Cytocentrifuge preparations of eosinophils incubated for 30hours at 37°C in medium alone (control), TNFα (10ng/ml), Mg132 (20μM), and combination of these reagents. The rates of apoptosis in this experiment are control 14.6 %, TNFα, Mg132 11.4% and MG132/TNFα 75.7%.

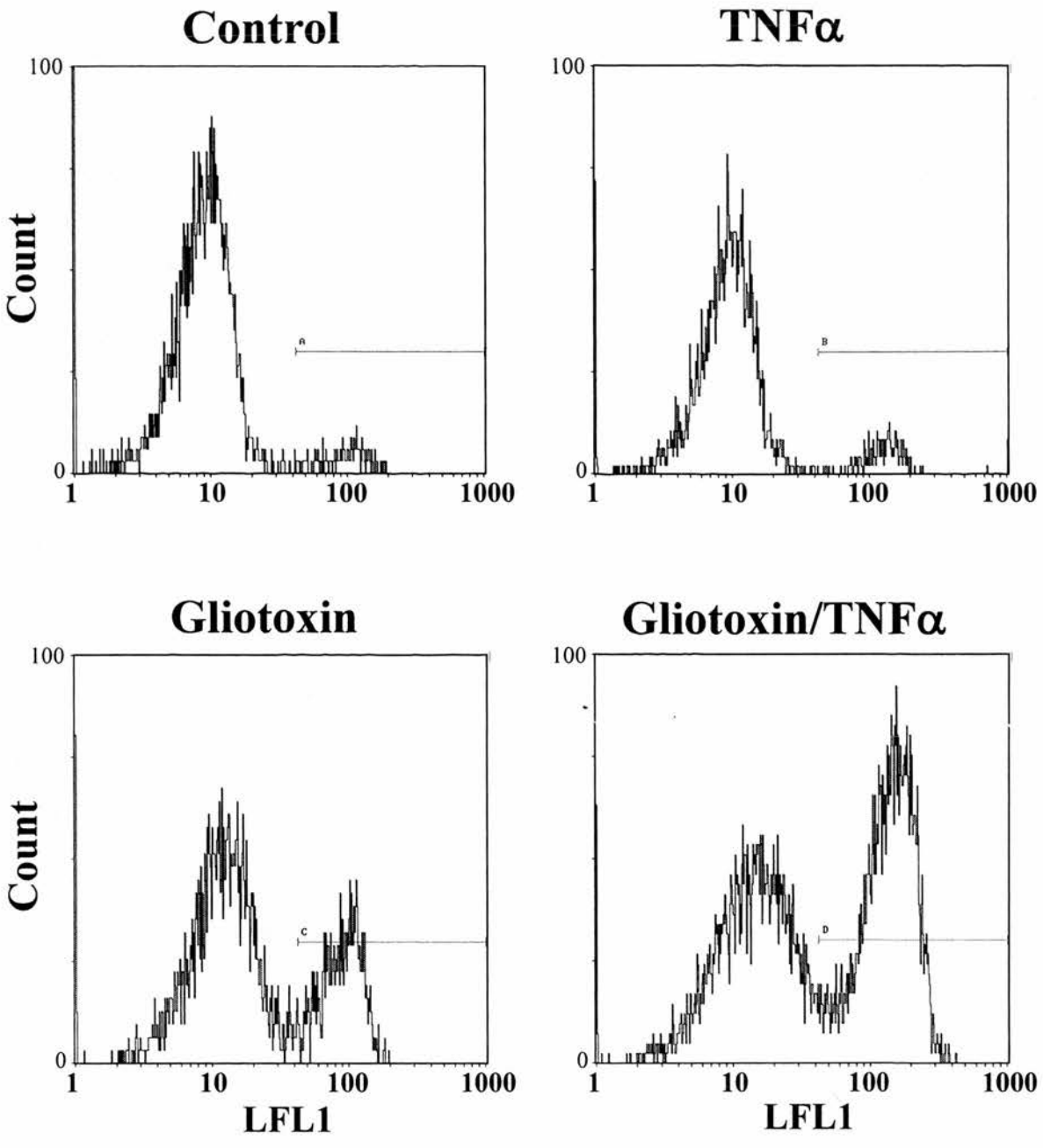


Figure 4-5-C; The effect of gliotoxin and TNFα on eosinophil apoptosis. Eosinophils were incubated for 4 hours at 37°C in medium alone (control), TNFα (10ng/ml), gliotoxin (100ng/ml), and a combination of these reagents and incubated with FITC- labelled recombinant human annexin V to determine the phosphatitylserine expression on the cell surface. Flow cytrometric traces from a typical experiment are depicted with the percentage of cells in the annexin-V high population being 7.0% in control, 9.8% in TNFα, 28.5% in gliotoxin and 50.8% in TNFα plus gliotoxin treated samples.

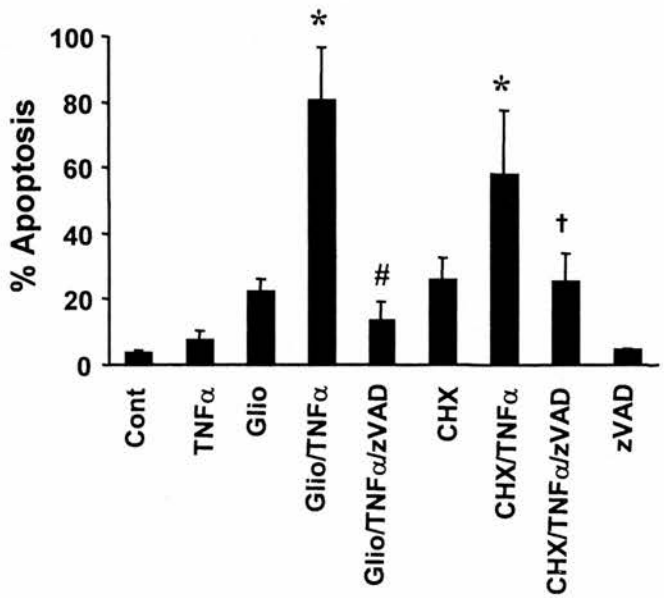


Figure 4-6; Effects of the caspase inhibitor, zVAD-fmk, and the protein synthesis inhibitor, cycloheximide, on eosinophil apoptosis induced by gliotoxin, TNFα or on a combination of these agents. Eosinophils were treated with the indicated reagents for 4 hours at 37°C before cytocentrifuge preparations were made and apoptosis assessed morphologically. Concentrations are as follows; TNFα (10ng/ml), gliotoxin (glio) (100ng/ml), zVAD-fmk (100μM), and cycloheximide (CHX) (5μM). The data are expressed as the mean ± SEM of 4 separate experiments using eosinophils isolated from different donors. *Represents significance differences ($p<0.05$) from control and # represents significance difference from Glio/TNFα and † represents significance difference form CHX/TNFα.

B

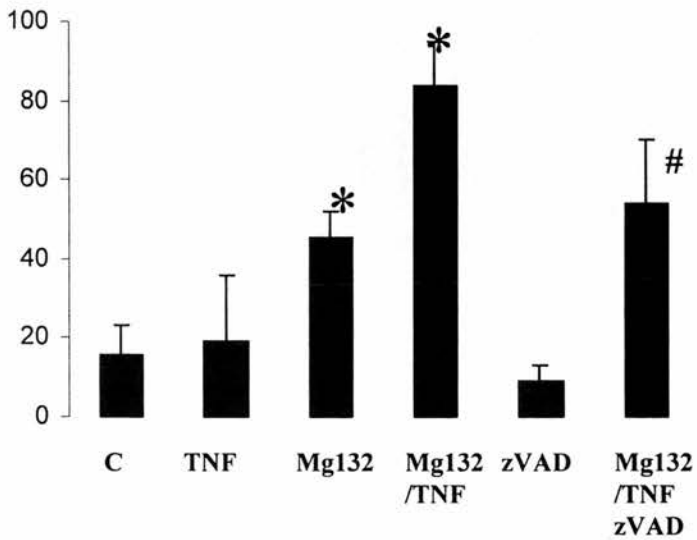


Figure 4-6-B; Effects of the caspase inhibitor, zVAD-fmkapoptosis induced by Mg132, TNFα or on a combination of these agents. Eosinophils were treated with the indicated reagents for 48 hours at 37°C before cytocentrifuge preparations were made and apoptosis assessed morphologically. Concentrations are as follows; TNFα (10ng/ml), Mg132 (100ng/ml), zVAD-fmk (100μM). The data are expressed as the mean ± SEM of 2 separate experiments using eosinophils isolated from different donors. *Represents significance differences ($p<0.05$) from control and # represents significance difference from Mg132/TNFα.

observe the synergistic effects of TNFα and gliotoxin. (Less than 4 hours, apoptosis rate is not significant.)

Similarly, panel B shows the effect of Mg132 on eosinophil apoptosis assessed morphologically. To observe the effect of Mg132 to induce TNFα-mediated eosinophil apoptosis, a time point of 30 hours was chosen. However, as it is shown in figure 4-5-B, apoptosis was significantly induced with co-culture of Mg132 and TNFα.

As an additional independent assessment of eosinophil apoptosis we performed experiments where surface changes associated with the apoptotic program were also assessed. For this we measured FITC labeled annexin V binding in the presence of Ca^{2+} to phosphatidylserine molecules exposed on the outer membrane of apoptotic cells where the annexin V 'low peak' represents non-apoptotic cells and the annexin V 'high peak' represents apoptotic cells. In agreement with morphological assessment of apoptosis, figure 4-5-C shows that although control and TNFα treated eosinophils at 4 hours exhibit low rates of apoptosis, the small increase in annexin V positive cells observed with gliotoxin alone is again dramatically augmented when the cells are cultured in the presence of both reagents together.

Furthermore, the marked synergism that was displayed by gliotoxin and TNFα is mediated by triggering of the caspase pathway since the broad-spectrum caspase inhibitor, zVAD-fmk (100 μM) completely blocked the pro-apoptotic effects induced by the combination of TNFα plus gliotoxin (figure 4-6). When eosinophils were co-cultured with the protein synthesis inhibitor cycloheximide (5μM), at a

concentration and time point that had almost no direct effect on eosinophil apoptosis, a similar un-masking of the pro-apoptotic effect of TNFα was observed. The pro-apoptotic effects of Mg132 *per se* and the enhanced apoptosis observed with a combination of Mg132 and TNFα was also suppressed by zVAD-fmk (figure 4-6-B).

We did not determine the possibility of other pathway such as PKC or cAMP, which may influence apoptosis pathway.

4.6. Discussion

In eosinophils, Western blot analysis and immunofluorescence data demonstrate that p65 translocates from the cytoplasm to the nucleus in response to TNFα. This action was prevented by gliotoxin and Mg132. Therefore, combining gliotoxin or Mg132 with TNFα enhances pro-apoptotic property of TNFα. Such conditions result in dramatic synergistic induction of apoptosis.

NF-κB binds to DNA regulatory sites on the upstream promotor sequences of target genes that control the rate of gene transcription for many pro-inflammatory mediators, some of which can influence granulocyte function and apoptosis (e.g., TNFα and IL-8). Eosinophils exposed to TNFα (figure 4-2-4) and LPS (Takanashi *et al.*, 1994) are capable of releasing IL-8. IL-8 itself can cause recruitment of inflammatory cells into sites of inflammation and can also prime and/or activate granulocytes (Baggiolini *et al.*, 1992, Harada *et al.*, 1994). Thus activation of the NF-κB pathway can augment the inflammatory response by generating further pro-inflammatory mediators. In our studies, synthesis of IL-8 is inhibited by gliotoxin even at a time point (90 min) where there is no significant induction of apoptosis, consistent with the observation that GM-CSF and TNFα activate NF-κB to induce IL-8 production in human eosinophils (Yamashita *et al.*, 1999).

The mechanism of inhibition of NF-κB by gliotoxin is unknown. Although we have shown previously that gliotoxin, but not its structurally similar analog, methylthiogliotoxin, can directly induce neutrophil apoptosis and enhance apoptosis in the presence of TNFα (Ward *et al.*, 1999) by selectively inhibiting NF-κB activation, the underlying mechanisms were not identified.

However, as it has been shown in figure 3-10 (chapter 3) in neutrophils, gliotoxin prevented TNFα- or LPS-induced IκBα degradation. The treatment with gliotoxin does not change the formation of IκBα, such as phosphorylation or ubiquitination. Gliotoxin may interrupt the phosphorylation pathway of IκBα, IKK complexes. IKKs are known to dimerised by disulfide bonds in helix-loop-helix (HLH) motifs (Mercurio *et al.*, 1997). Mutation in HLH motifs of IKK1 and IKK2 result in loss of kinase activity (Zandi *et al.,* 1998). Gliotoxin is known to disrupt the interaction of disulfide bonds (Waring and Beaver, 1996). Therefore, gliotoxin might disrupt the disulfide composition of IKKs resulting in no phosphorylation on IκBα (This idea was kindly provided by Dr James Matthews, University of Wales). This might explain the gliotoxin induced inhbition of NF-κB in various cell types and cause the gliotoxin mediated immunosuppression and apoptosis in inflammatory cells. This interesting possibility was not investigated further.

Interestingly, Mg132, like gliotoxin, can also induce eosinophil apoptosis directly and also renders eosinophils responsive to the pro-apoptotic effects of TNFα. It is noteworthy that Mg132 required a longer incubation period in comparison to gliotoxin to unmask TNFα induced apoptosis. The precise reason for this is unknown but may reflect a difference in the mechanism of action or in the rates of cellular incorporation between the two inhibitors of NF-κB activation.

TNFα triggers a number of signalling pathways following ligation of TNFα receptors. We have previously shown that neutrophils undergo an early induction of apoptosis (2-8 h) and a later (>18 h) inhibition of apoptosis when cultured *in vitro* in

the presence of TNFα (Murray et al., 1997). The pro-apoptotic effect of TNFα is thought to be mediated by ligation of the TNFR1 containing a death domain that interacts with the TNFα-receptor-associated death domain protein (TRADD) allowing recruitment of Fas-associated death domain (FADD) leading to activation of the caspase enzyme pathway. This highly regulated sequence of events enables apoptosis to be triggered. TNF receptor 2, often in co-operation with TNFR1, results in the activation of many kinases including the p38-Jun N-terminal kinase (JNK) pathway and IKK (Rath and Aggarwal, 1999). It remains possible that blockade of NF-κB will prevent the synthesis of NF-κB regulated survival cytokines such as GM-CSF, IL-5 and IL-3. Indeed, it has recently been published that TNFα via ligation of both TNFR1 and TNFR2 subtypes mediated eosinophil survival (as assessed by Trypan blue exclusion) and that this occurred through regulation of GM-CSF but not IL-3 and IL-5 production (Temkin and Levi-Schaffer, 2001). Furthermore, it was shown that GM-CSF is indeed synthesized when eosinophils are challenged with TNFα and that TNFα enhanced eosinophil survival is significantly inhibited by the proteasome inhibitor Mg132 (Temkin and Levi-Schaffer, 2001). It also remains likely that eosinophils like other cells can generate protein(s) that directly influence the apoptotic program. However, the precise identity of the potential survival protein(s) is unknown, however several likely candidate proteins have been implicated including members of the oncogene Bcl2 family such as A1 (Chuan *et al.*, 1998, Hamasaki *et al.*, 1998) and Mcl-1(Moulding *et al.*, 1998) and others such as c-Myc (Evan *et al.,* 1992), A20 (Sarma *et al.*, 1995), cIAP (Chu *et al.*, 1997, Wang *et al.*, 1998), XIAP, c-IAP1, c-IAP-2 (Stehlik *et al.,* 1998, Deveraux *et al.,* 1997, Deveraux *et al.,* 1998), and IEX-1L(Wu *et al.*, 1998).

Although it is abundantly clear that NF-κB activation can play an important role in regulating the production of cytokines and adhesion molecules that are vital for orchestrating the inflammatory response there is relatively little direct *in vivo* evidence indicating that this transcription factor can influence eosinophilic inflammation. Compelling evidence that NF-κB plays an essential role in the induction of eosinophilia in allergic airway inflammation, was obtained using mice deficient in p50, when compared with wild type mice (Yang et al., 1998). In addition to being incapable of mounting eosinophilic airway inflammation, p50 -/- mice had a dramatically reduced capacity for the production NF-κB regulated inflammatory mediators such as the T helper 2 cytokine IL-5 and the chemokines eotaxin and MIP-1.

In summary, these data strongly suggest an important role of NF-κB in controlling eosinophils responsiveness and apoptosis.

Chapter 5
Use of TAT-IκBα for the inhibition of NF-κB in HeLa, A549 cells and eosinophils, and its effect on eosinophil apoptosis

5.1. Introduction

5.1.1. TAT protein and its domains for transduction

The HIV-1-trans-activator gene product, TAT, has been shown to be critical for transduction of virus into cells and could transfect the viral HIV-LTR (Long Terminal Repeat) promotor to cause virus replication. This 86 amino acid protein has been studied by several groups for transduction ability into the both the cytoplasm and nucleus. In 1988, for the first time, Green *et al.* (1988) and Frankel *et al.* (1988), independently showed that TAT (amino acid 37-72), could transduce into the cells. After their discoveries other groups have demonstrated that various different parts of TAT have ability to transduce (see table 5-1-1), for example, TAT amino acid (a.a.) 38-58 (Mann *et al.*, 1991), a.a. 1-72, 37-72 (Farwell *et al.*, 1994), a.a. 37-47 (Vives *et al.*, 1997) and a.a.47-57 (Nagahara *et al.*, 1998). Furthermore, Dowdy and co-workers (Nagahara *et al.*, 1998) have demonstrated that the arginine rich motif (ARM), which corresponds to amino acids 47-57, has the most efficient transduction ability. ARM contains nucleus localisation signals (NLS) and RNA binding domains to allow virus to manipulate transcriptional activities when the rest of the virus is presented (Friedler *et al.*, 2000). Thus, ARM is the most important part for HIV-1 for virus internalisation and to promote transcription activity for virus replication.

Distinct from TAT, a similar transduction ability has been demonstrated by the protein transduction domain (PDT) by VP22 from herpes simplex virus (Elliott and O'Hare 1997), and the Drosophila Antennapedia homeotic protein (Joliot *et al.*, 1991), which also possesses a similar high arginine and lysine content. Both transduction proteins may share a similar mechanism and function as TAT (see

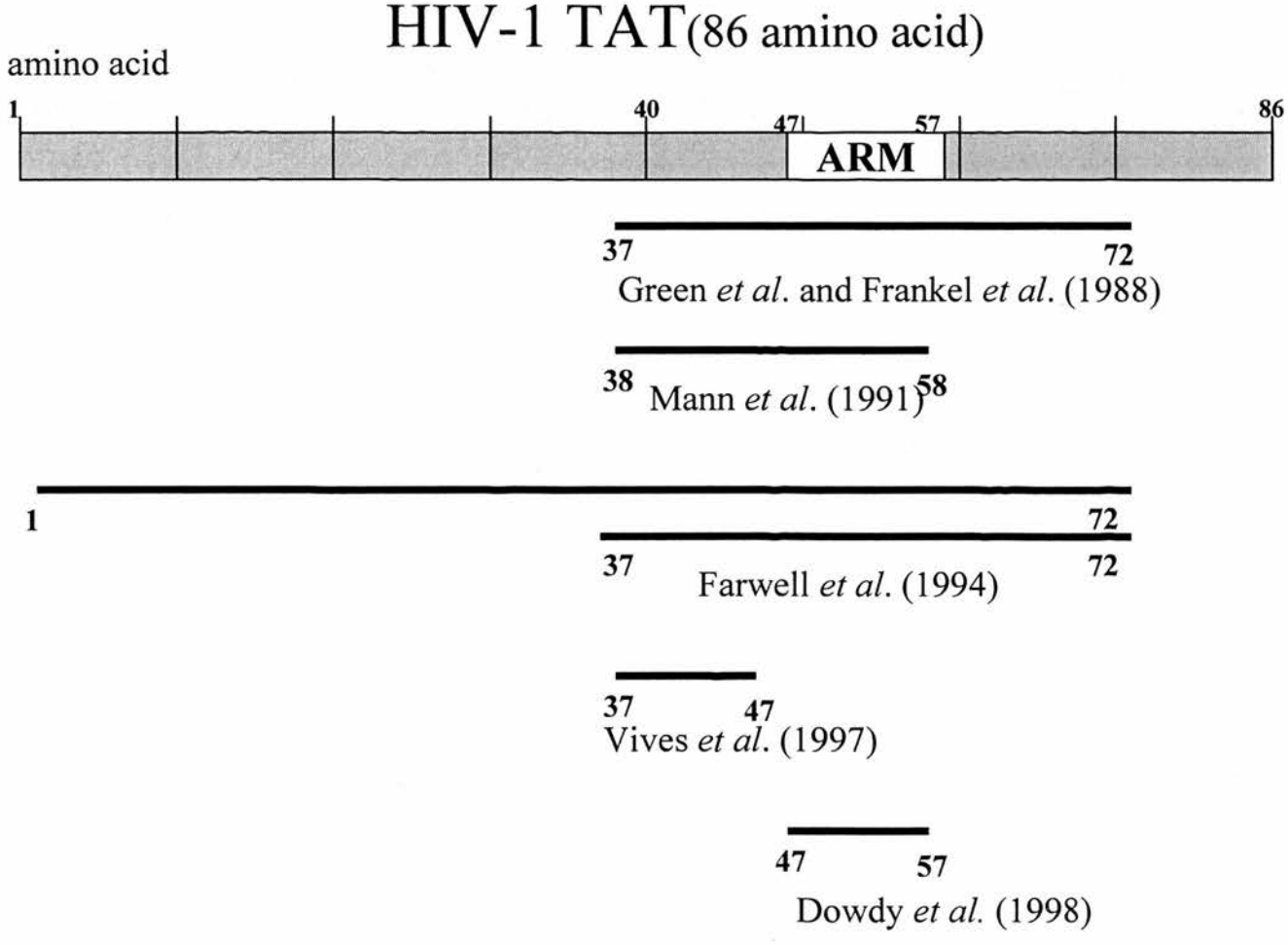


Figure 5-1-1; Summary of reported transduction domains in 86 a.a. HIV-1 TAT. Various groups have demonstrated efficient transduction domains in TAT. Arginine rich motif (ARM) has been identified as the most effective transduction domain in TAT.

5.1.3.).

5.1.2. TAT as a molecular tool

Farwell *et al.*, (1994) first demonstrated that TAT transduced not only HIV-1 virus but also some other proteins linked with TAT through the cell membrane. β-galactosidase linked with TAT (a.a. 1-72, 37-72) could be successfully transduced into various tissues and organs in mice. Nagahara and Dowdy (Nagahara *et al.*, 1998) developed an 11 amino acid ARM TAT (GYGRKKRRQRRRG) as a 'tool' to transduce proteins such as antibodies and enzymes, nucleic acids and beads into cells in a receptor and transporter independent manner. Furthermore, Dowdy and co-workers (1998, 1999, 2000a, 2000b) demonstrated that proteins linked to TAT such as TAT-p27 (Nagahara *et al.*, 1998), TAT-caspase3 (Vocero-Akbani *et al.*, 1999), TAT-GTPase (Vocero-Akbani *et al.*, 2000), TAT-β-galactosidase (Schwarze *et al.*, 2000), resulted in 100% efficiency transduction in 30 minutes into many cells (e.g., lymphocytes, diploid human fibroblasts, keratinocytes, bone marrow stem cells, osteloclasts, fibrosarcoma cells, osteosarcoma, glioma, hepatocellular carcinoma, renal carcinoma, NIH 3T3). Similarly other groups have been demonstrated TAT transduction into various cell types using different proteins linked with TAT (for a summary see table 5-1-2). Farwell *et al.* (1994) and Pooga *et al.* (1998) have shown that almost any type of tissue can be transfected by TAT, including the blood-brain barrier. In granulocytes, Hall *et al.* (2000) and Alblas *et al.*, (2001) and Bruyninckx *et al.*, (2001) have demonstrated that TAT fusion protein is successfully transduced into eosinophils and neutrophils which are generally extremely difficult to molecularly manipulate. For example, the efficiency of transduction of TAT-H-Ras-FITC in eosinophils was almost 100% in 30 minutes

Hall (2001)	TAT-dn-H-Ras	eosinophils
Bruyninckx (2001)	TAT-β3-intergrin ctytoplasmic tail	neutrophils
Dabouridis (2002)	TAT-IκBα	HeLa and Jurkat cells
Nagahara, Dowdy (1998)	TAT-p27 TAT-Cdk2 (DN)	peripheral blood lyphocytes, diploid human fibroblasts, keratinocytes, bone marrow stem cells, osteoclasts, fibrosarcoma cells, osteosarcoma, glioma, hepatocellular carcinoma, renal carcinoma, NIH 3T3
Vocero-Akbani, Dowdy (2000,1999,2001)	15 to115KDa TAT fusion protein TAT-caspase3 TAT-GTPase	Peripheral blood lymphocytes, all cells present in whole blood, bone marrow stem cells, diploid fibroblasts, fibrosarcoma cells, keratinocytes
Schwarze, Dowdy (2000)	1. TAT-E1A 2. TAT-Rho 3. TAT-βgal	1. lymphocyte, peripheral blood mononuclear cells, 2. osteoclast culture 3. many tissues in mice, including brain, bone marrow etc.
Abu-Amer (2001)	TAT-IκBα42	Osteoclast precursors
Jin (2001)	TAT-CAT 9Arg-CAT	HeLa, PC12 cells
Pooga (1998)	Antp-anti-sense-peptide-nucleic-acid oligomers	blood-brain barrier in rat
Fenton (1998)	Fluorescently labelled Antp peptide	lymphocytes
Brachmann (PC)	TAT-GFP	*Drosophila* eyes
Fawell (1994)	TAT-β-gal	Limited but significant capacity to tissues in mouse

Table 5-1-2; Summary of reported examples of TAT linked with various proteins, which transduced into different cell types.

pc;personal communications to Schwarze and Dowdy (2000).

(Hall *et al.*, 2000). This remarkable result should lead to further usage of the TAT system to transduce protein into eosinophils in order to investigate specific intracellular proteins and signalling pathways (see 5.1.4. for the usage of TAT in eosinophils).

In summary the advantages of the usage of the TAT-transduction technique are given as follows;

- Direct transduction of peptide, protein, nucleic acid and small objects (e.g. beads) into cells
- Most cells can be transduced (specific receptor and transporter independent mechanism)
- Rapid transduction (TAT internalisation sometimes starts from 5 minutes.)
- 100% efficient (different from virus transfection)
- Specific and direct approach to manipulate cell signalling pathways (different from using pharmacological agents which might influence other pathways).

Thus, TAT fusion proteins can provide an huge potential in molecular and cellular biology research, especially for cells which do not accept virus transfection methods.

5.1.3. The mechanisms involved in TAT transduction

The precise mechanism of translocation of TAT peptide through the membrane is not currently known. However, various groups have suggested possible mechanisms, for example; a) an energy endocytosis pathway, b) via low density lipoprotein receptor-related protein (LRP) and c) via a cell surface heparan sulfate (HS).

a) The energy endocytosis pathway; Recently Eguchi *et al.*, (2001) have reported that conserved domains of TAT is used by λ phage for its transduction into cells via caveolae on the cell surface. Caveolae are small flask-shaped invaginations of the plasma membrane, rich in cholesterol and glycosphingolipids, which forms coated buds for transportation. However, Suzuki *et al.*,(2002) showed that Nystain, a caveolae formation inhibitor did not suppress TAT (48-60), Rev (34-50; TRQARRNRRRRWRERQRGC) and arginine rich peptides (Arg8) (RRRRRRRR-GC) intake into cells. Also, other endocytosis inhibitors and metabolic inhibitors did not show any suppression of TAT and other arginine rich peptide intake. Thus, energy endocytosis may be involved but not crucial for TAT transduction system.

b) The low density lipoprotein receptor-related protein (LRP); Liu *et al.*, (2000) have demonstrated that TAT segments (32-48), (48-57) are transduced into neurons through lipoprotein receptor-related protein (LRP). However, LRP expression varies in different cell types and organs and so it does not explain why TAT peptide transduction is able to occur in almost all cell types as described previously (Nagahara *et al.*, 1998). However, Suzuki *et al.* (2002) claimed that internalisation of TAT domain (48-60), Rev (34-50) and arginine rich peptides (Arg8) were not affected by disturbance of LRP. Thus, LRP may not play a major role in the transduction of TAT peptide or arginine rich peptide.

c) Cell surface heparan sulfate (HS); heparan sulfate (HS) is strongly negatively charged and expressed ubiquitously on the surface of animal cells. Negatively

charged HS may cause electrostatic interaction with positively charged arginine rich peptide ARM in TAT. So ARM may act as 'cargo' to flick over whatever the proteins or objects are linked to TAT into the cells. Suzuki *et al.*, (2002) has shown that anti-HS antibody or blocking the function of sulfated polysaccharides significantly inhibited internalisation of TAT (48-60), Rev (34-50) and arginine rich peptides (Arg8). Thus, this strongly suggests that sulphated polysaccharides of HS is a favourable explanation for TAT internalisation.

Although, as it has been reported by Suzuki *et al,* (2002) that the slight differences in experimental methodologies (e.g. full length of TAT or TAT peptides) may give contradictory explanations or that more than one mechanism is involved in TAT internalisaion.

5.1.4. The possibility of TAT trasduction system in eosinophils

For the introduction of specific cellular proteins into cells, manipulation by viral transfection and microinjection have been demonstrated to be useful techniques in various cell types, often in stable cell lines. However, eosinophils are particularly difficult cells in which to perform such experiments due to lack of virus receptors or specific transporters and because of their life span. Thus, much eosinophil study is dependent upon the usage of pharmacological reagents which may be limited by their specificity. Therefore, we decided to explore the usage of TAT to introduce cellular proteins into eosinophils for the first time as a direct approach to manipulate intracellular signalling pathways.

During this project, Hall *et al.* (2001) have demonstrated transduction of TAT-p27

into eosinophils. They showed that within 30 minutes, FITC-TAT-RAS and FITC-TAT-GFP were transduced into 95% of the eosinophils. This remarkable result shows that even eosinophils are capable of using this TAT transduction system, and opens the opportunity for a direct approach to understand the mechanisms and functions of specific cellular proteins in eosinophils.

5.1.5. TAT-IκBα

Various groups have reported that TAT can be linked with various proteins including IκBα. Abu-Amer *et al.,* (2001) have used TAT-IκBα (mutated at tyrosine 42) to study the function of tyrosine cascades in osteoclastogenesis. In these experiments bone marrow cells are also difficult to transfect, but were successfully transduced by TAT-IκBα and the resulted in the arrest of osteoclastogenesis. Recently, Kabouridis et al., (2002) have also demonstrated inhibition of IL-1- and TNFα-induced-NF-κB by TAT-IκBα32,36 in HeLa and Jurkat cells. These reports suggest the possibility of the usage of TAT-IκBα as a tool to manipulate the NF-κB signalling pathway in cell lines and bone marrow cells.

Once cells receive an extracellular stimuli, such as TNFα, IL-1, LPS, through the IKK complex, IκBα is phosphorylated on serines 32 and 36 in its N-terminus and is ubiquitinated. This form of IκBα is then recognised and degraded by the 26S proteasome. When NF-κB is freed upon IκBα degradation, NF-κB translocates into the nucleus for the induction of transcription and protein synthesis. Prevention of IκBα degradation results in the inhibition of NF-κB. Therefore, mutation of the phosphorylation sites at Ser 32 and Ser 36 prevents IκBα degradation and over-expression of IκBα in cytoplasm results in prevention NF-κB translocation into the

nucleus. Mutation of these phorphylation sites has been a most favoured approach for inhibition of NF-κB by viral transfection or by microinjection methods.

When TAT-IκBα is transduced into cells through the cell membrane, it should over-express IκBα and prevention of NF-κB into the nucleus for transcriptional activities. This method may overcome some disadvantages of using pharmacological reagents and viral transfection. Here, TAT-IκBα both wild type (WT), mutation of Ser 32 and Ser 36 (TAT-IκBα32, 36) and GST-TAT as a control, were generated to investigate NF-κB inhibition in four different types of cells, namely HeLa, A549, Jurkat cell lines and eosinophils.

In our previous study (see chapter 4 and Fujihara *et al.*, 2002) inhibition of NF-κB in eosinophils was demonstrated to be the one of the key elements regulating apoptosis upon TNFα stimulation. Inhibition of NF-κB was dependent on pharmacological reagents, such as Mg132 or gliotoxin. Here, using the TAT system, the mechanism of NF-κB pathway to induce apoptosis in eosinophils is further investigated.

The aims of this chapter are;

- To introduce methodology of cloning of TAT-IκBα.
- To investigate the effect of TAT-IκBα in HeLa cells, A549 cell lines and eosinophils.
- To investigate the effect of TAT-IκBα on eosinophil apoptosis.

5.2. - 5.5. Results

5.2. Cloning and purification methods for TAT-IκBα

5.2.1. Cloning map

As shown in figure 5-2-1, GST-TAT-IκBα gene was cloned for TAT-IκBα wild type (WT), TAT-IκBα mutated at S32,36, and recombinant wild type of IκBα (rIκBα) and GST-TAT. (See Methods and Materials in chapter 2 for more detailed methods.) GST-TAT, which had been obtained from the same plasmid has been used in the following experiments as a control protein for TAT-IκBα.

5.2.2. DNA Sequence

After the insertion of the TAT and IκBα gene into pGEX2T and transformed into *E.Coli* DH5α cells, some colonies were selected for DNA sequencing, PCR (data not shown) and mini protein purification (see figure 5-2-3) to determine the gene which contains the TAT-IκBα. Typical results of a successful DNA sequence of GST-TAT-IκBα gene is shown in figure 5-2-2. TAT sequence (a.a.GYGRKKRRQRRRG) is highlighted in **bold** letters. Combining all the results of DNA sequencing, PCR and mini protein purification, a successful gene (colony) was chosen for protein purification as described in the method section.

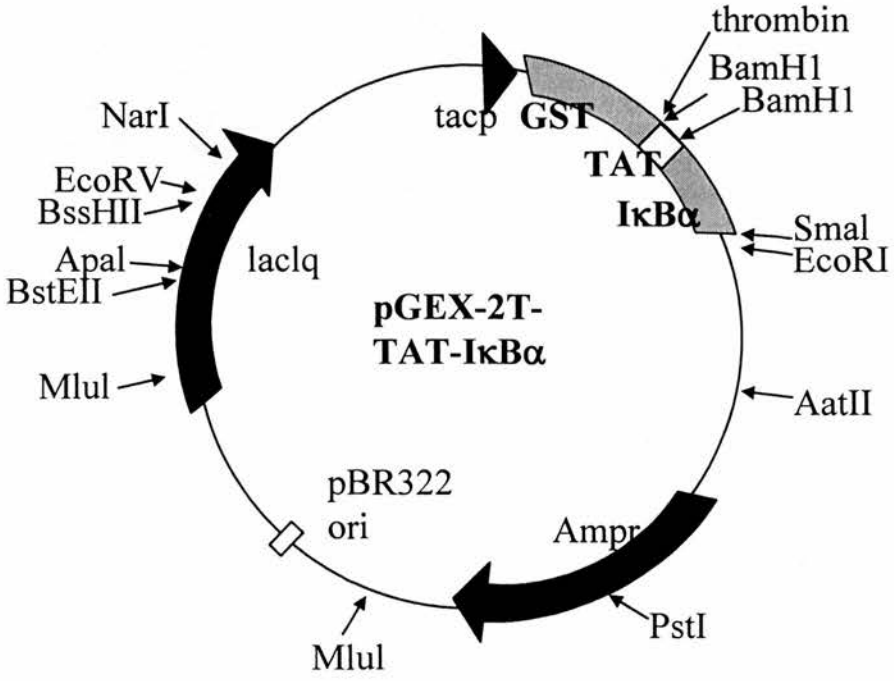
Figure 5-2-2; DNA sequence for TAT, reading bottom strand.

5'...(IκBα)....CGCTGGAACATG**GATCCACGGCGACGCTGG**

CGACGTTTTTTGCGGCCATAAGATCCACNCGGAACCANA

TCCGATTTTGGAGGATGG...(GST)..3'

A



B

DNA or protein	thrombin	post-purification
GST-TAT-IκBα	+	TAT-IκBα
GST-IκBα	+	IκBα
GST-TAT	-	GST-TAT

Figure 5-2-1;A. Plasmid map of pGEX-2T inserting TAT and IκBα gene. IκBα and TAT were inserted into pGEX-2T at the GST site. **B. DNA or protein for protein purification**; For protein purification, thrombin was used to cut off GST parts and eluted through a column (see methods section, in chapter 2).

5.2.3. Protein purification

After protein purification (as described in the method section), the protein from the peak fraction was run on a 10% acrylamide gel for determination of TAT-IκBα by molecular weight, compared to recombinant IκBα (37KDa). TAT-IκBα is 11 amino acids heavier than recombinant IκBα, thus this difference is shown as a shifted band on the gel compared with recombinant IκBα. In figure 5-2-3 lane 1; recombinant IκBα, lane 2; TAT-IκBα are shown. 11 amino acids clearly show the band shifted in lane 2 compared with lane 1 with recombinant IκBα.

5.2.4. Mass Spectrometry

The purified TAT-IκBα protein in the peak fraction was also checked by mass spectrometry using slow crystrallisation (Botting, 2000) by Dr Botting, University of St Andrews, as shown in figure 5-2-4. In the figure 5-2-3 on the protein gel TAT-IκBα was shown to be the shifted band and was analysed by mass spectrometry as a molecular weight of 37579Da, whereas recombinant IκBα is 35471Da

5.3. Inhibition of NF-κB by TAT-IκBα (WT and 32,36) in HeLa cells

5.3.1. Controls; recombinant IκBα and GST-TAT in HeLa cells

As described previously (Arenzana-Seisdedos *et al.*, 1997) a cloned HeLa cell line (57A), containing luciferase gene linked with NF-κB binding sites was used to determine NF-κB activation. Briefly, these cells were transfected with luciferase gene down stream of the NF-κB promoter regions. Therefore, NF-κB activation is

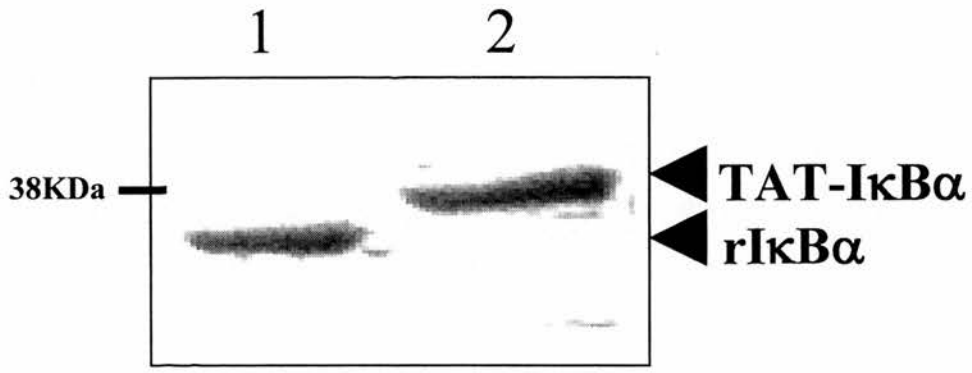


Figure 5-2-3; TAT-IκBα and recombinant IκBα (rIκBα). TAT-IκBα and r IκBα were run on a 10% polyacrylamide gel. TAT-IκBα is shown to be heavier than 37KDa recombinant IκBα (rIκBα).

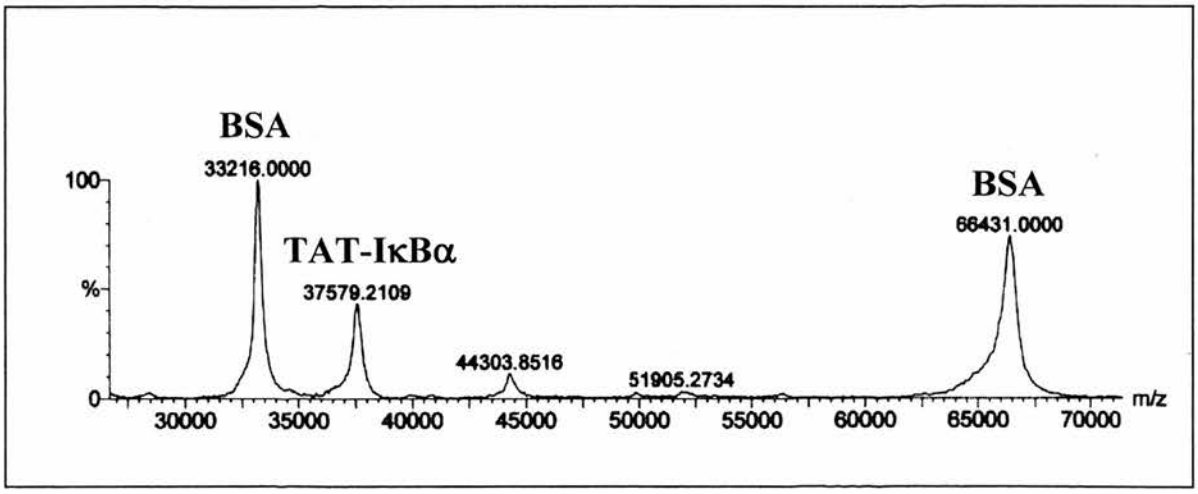


Figure 5-2-4; Analysis of TAT-IκBα by mass spectrometry. The molecular mass of TAT-IκBα was measured by mass spectrometry by slow crystallisation and calibrated with BSA by Dr Botting (University of St Andrews).

correlated with the amount of luciferase synthesised. GST-TAT and recombinant IκBα were both made using the same methods as TAT-IκBα and were used as control proteins to determine NF-κB activation with or without TNFα. GST-TAT (30μg/ml) and recombinant IκBα (rIκBα) (30μg/ml) were pre-incubated with cells for 30 minutes at 37°C before TNFα (5ng/ml) was added. After 6 hours of incubation, cells were harvested for luciferase measurement (see chapter 2 for further details in methods).

Figure 5-3-1-A shows the effect of luciferase activation in HeLa cells when recombinant IκBα (rIκBα) is incubated with or without TNFα. There was no significant effect on rIκBα both with or without TNFα compared to control conditions without rIκBα. Recombinant IκBα did not induce any activation nor inhibition on its own and did not inhibit TNFα -induced NF-κB activation.

GST-TAT was used as another control protein for TAT-IκBα. Figure 5-3-1-B shows the effect of GST-TAT. There was slight activation (14-fold) observed when GST-TAT was used. Similarly, stimulated with TNFα, GST-TAT induced luciferase activation (164-fold). This effect was greater than TNFα on its own (137-fold).

Summary

- rIκBα does not influence luciferase activity in HeLa cells with or without TNFα.

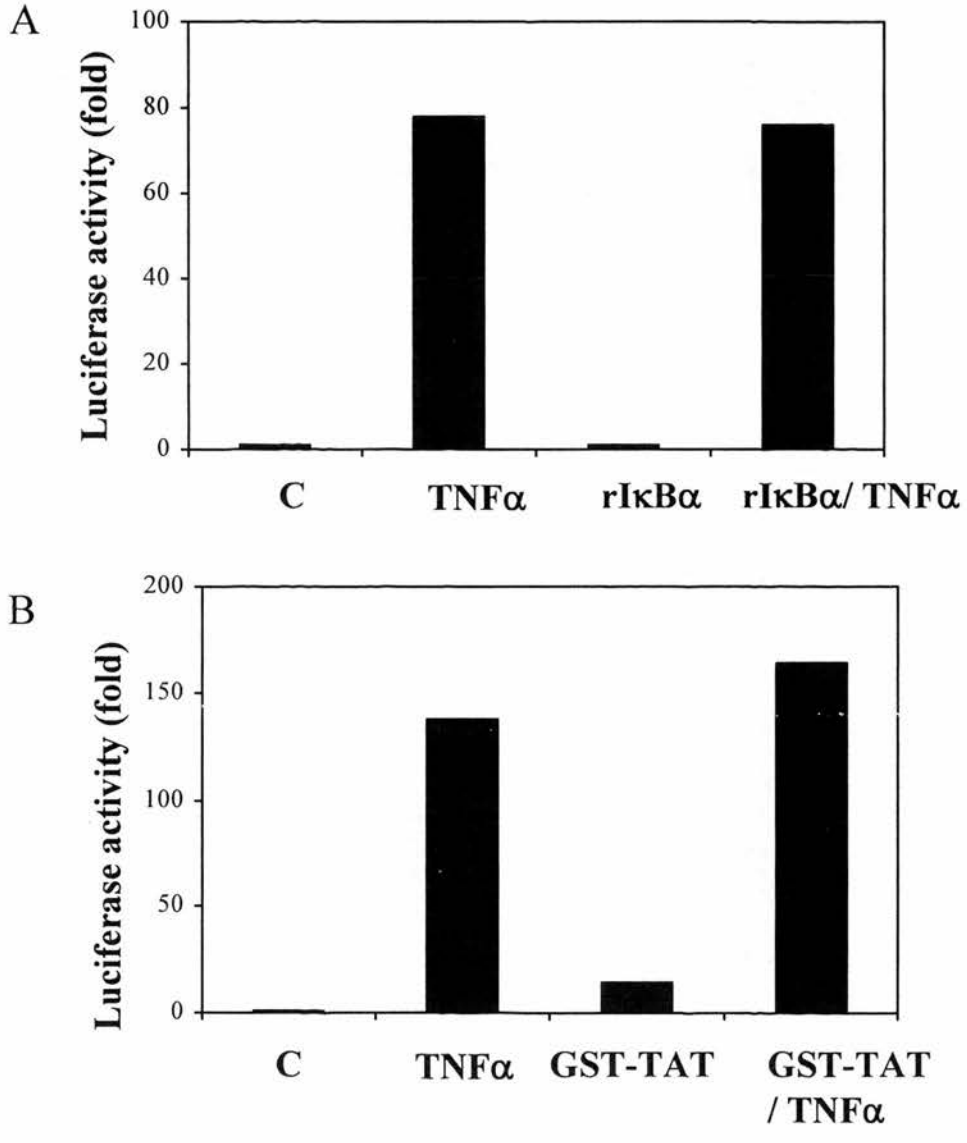


Figure 5-3-1; Recombinant IκBα and GST-TAT do not inhibit TNFα-induced NF-κB activation in HeLa cells. HeLa cells containing NF-κB-dependent the luciferase reporter gene was incubated with recombinant IκBα (rIκBα) (30μg/ml) (A) or GST-TAT (30μg/ml) (B) for 30 minutes at 37°C and treated with TNFα (5ng/ml). All the conditions were incubated for a further 6 hours for luciferase expression. 'C' indicates the control condition, which had no treatment. The activation of luciferease were measured and was compared with untreated condition 'C' and expressed as fold (Y-axis). **A.** recombinant IκBα does not induce NF-κB activation nor inhibit TNFα-induced activation. **B.** GST-TAT itself induces NF-κB activation slightly and further induction is seen when TNFα is added. This is a representative result of triplicate experiments performed on at least two separate occasions.

- GST-TAT does slightly increase luciferase activity in HeLa cells whether alone or in the presence of TNFα.

5.3.2. TAT-IκBα (WT and 32,36) in HeLa cells

Purified TAT-IκBα32,36, and TAT-IκBαWT (wild type), were assessed for their effects on NF-κB activation in HeLa cells. TAT-IκBα32,36 has serines 32 and 36 on N-terminus mutated with alanine, resulting in the prevention of phosphorylation of IκBα and over-expression of IκBα in the cytoplasm. TAT-IκBα32,36 and TAT-IκBαWT were incubated with cells for 30 minutes at 37°C before adding TNFα. Then the cells were measured for luciferase activity.

Figure 5-3-2-A demonstrates that TAT-IκBα32,36 significantly inhibits TNFα-induced NF-κB activation. TAT-IκBα32,36 on its own from 0.03 μg/ml to 100 μg/ml, did not significantly affect luciferase activity compared with untreated control cells (indicated as 'C'). In this experiment TNFα on its own induced transcriptional activity and was about 81-fold compared with control (C). However, this TNFα-induced-NF-κB-activation was inhibited by incubation with TAT-IκBα32,36. There was a dose-dependent inhibition of TNFα-induced NF-κB activation. For example, luciferase activity was about 5 fold at 100 μg/ml TAT-IκBα32,36 with TNFα. This is about 93% inhibition compared with TNFα only (81-fold). Similarly, 91% inhibition of TNFα-induced transcription was observed at 30μg/ml TAT-IκBα32,36. Even at 0.03μg/ml TAT-IκBα32,36 reduced TNFα-induced luciferase activity by 61%.

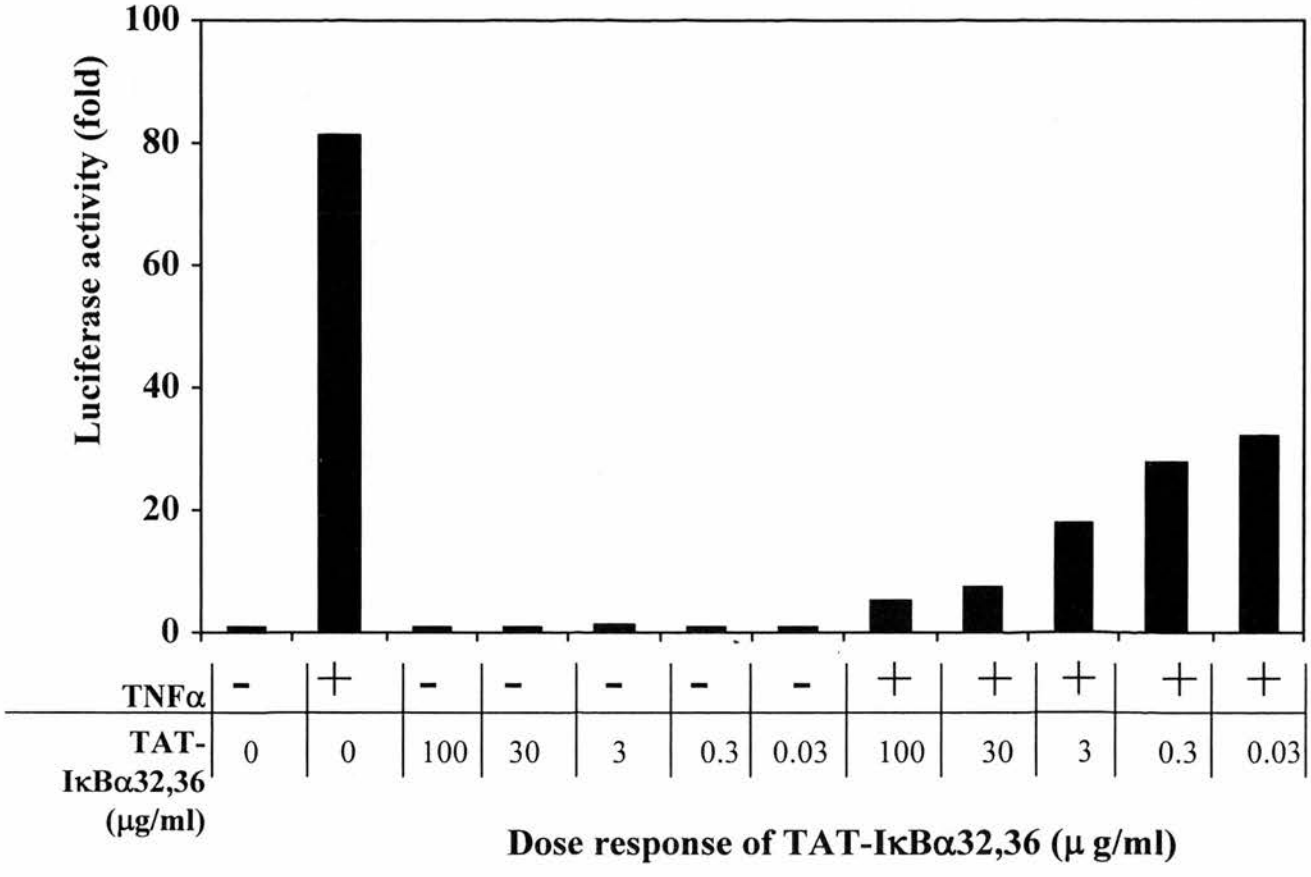


Figure 5-3-2-A; TAT-IκBα 32,36 inhibits TNFα-induced NF-κB activation in HeLa cells. HeLa cells containing NF-κB-dependent luciferase reporter gene was incubated with TAT-IκBα 32,36 for 30 minutes at 37°C and further treated with TNFα and then all the conditions were incubated for 6 hours for luciferase expression. The activation of luciferease was measured and was compared with untreated condition and expressed as fold (Y-axis). Lane 1 from the left. TAT-IκBα 32,36 itself does not influence luciferase activity, compared to no treated condition in lane 1 from the left. When TNFα is added, TNFα-induced NF-κB activation was suppressed by TAT-IκBα 32,36 in dose-dependent manner. The dose of TAT-IκBα 32,36 correlates with the inhibition of luciferase activity. This is a representative result of triplicate experiments performed on at least two separate occasions.

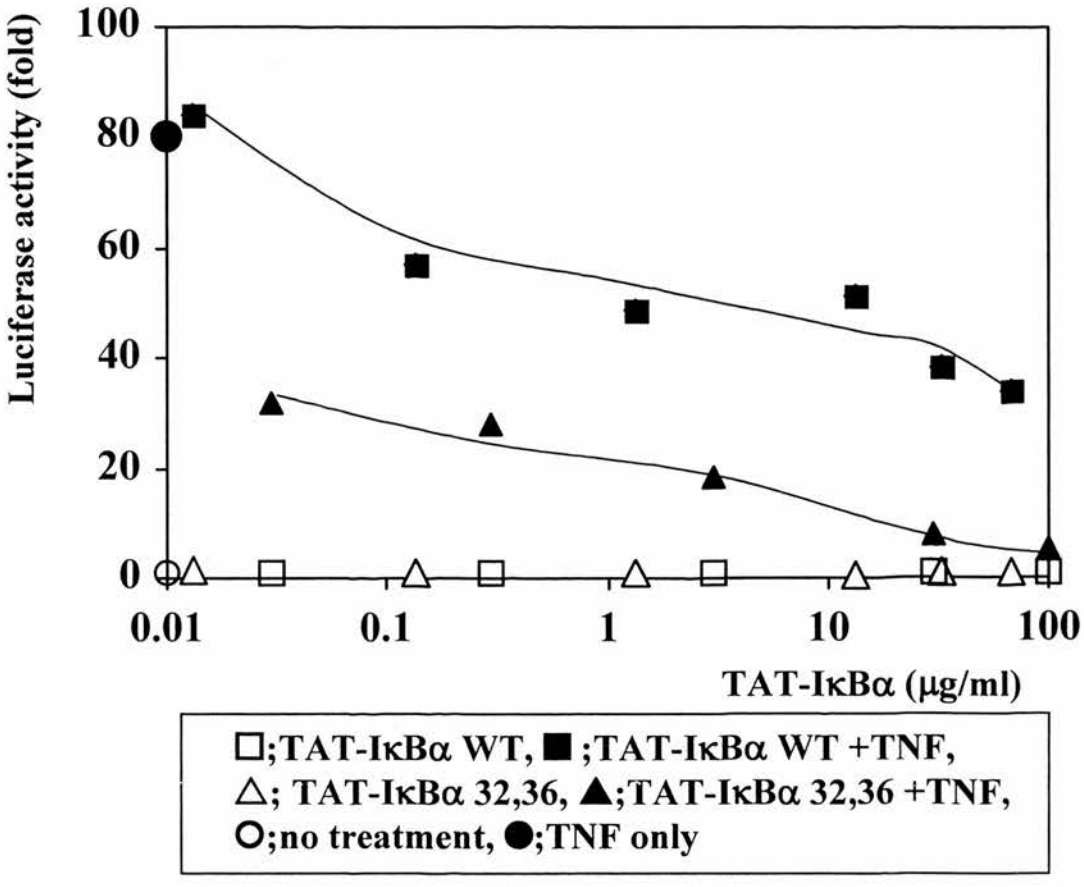


Figure 5-3-2-B; TAT-IκBα 32,36 inhibits TNFα-induced NF-κB greater than wild type (WT) TAT-IκBα. HeLa cells containing NF-κB-dependent luciferase reporter gene was incubated with TAT-IκBα 32,36 or TAT-IκBα wild type (WT) for 30 minutes at 37°C and further treated with TNFα for 6 hours for luciferase expression. The activation of luciferease were measured and was compared with untreated condition (O) and expressed as fold (Y-axis). '●' on Y-axis indicates the luciferase activity of TNF only. TAT-IκBα 32,36 shows lower luciferase activity than TAT-IκBα WT. In other words, TNFα-induced NF-κB transcriptional activity was more suppressed by TAT-IκBα 32,36, which is incapable of being phosphorylated, compared with TAT-IκBα WT, which can still be degraded upon TNFα stimulation. At the lower concentration TAT-IκBα WT reaches at the same transcription level as TNFα only ('●' on Y axis). This is a representative result of triplicate experiments performed on at least two separate occasions.

The different effects of inhibition of transcription activity of NF-κB by TAT-IκBα32,36 and TAT-IκBαWT is demonstrated in figure 5-3-2-B. Dose-response curves were performed in the presence of TAT-IκBα and TNFα. TNFα only (85-fold) is shown as filled circle on Y axis in the graph. There is no induction of luciferase activity seen by TAT-IκBα32,36 and TAT-IκBαWT alone.

Both TAT-IκBα32,36 and TAT-IκBαWT inhibit TNFα-induced NF-κB activation in a dose-dependent manner. There is a significantly different effect on the inhibition of TNFα-induced NF-κB activation between the mutation and wild type. Compared with TAT-IκBα32,36, TAT-IκBαWT is less effective at inhibiting TNFα-induced NF-κB activation. For example, the lowest dose of TAT-IκBαWT (0.013 μg/ml), had no effect on TNFα-induced transcription (83-fold). Wild type can still inhibit TNFα-induced NF-κB activation but its effect is limited. Although WT is over-expressed in the cells they are still capable of being degraded and as a result in to free NF-κB for transcriptional activities. On the other hand, TAT-IκBα32,36 is not able to be degraded, which therefore results in a strong inhibition. Therefore, TAT-IκBα32,36 was chosen to be used for the rest of the following experiments.

Summary

- TAT-IκBα32,36 and TAT-IκBαWT have no effect on NF-κB activation alone.
- TAT-IκBα32,36 inhibits TNFα-induced-NF-κB activation.
- TAT-IκBαWT inhibits TNFα-induced-NF-κB activation.

- TAT-IκBα32,36 is a stronger inhibitor of TNFα-induced NF-κB activation compared to TAT-IκBαWT.

5.3.3. Other experimental conditions

Because TAT-IκBα is a new technique, therefore, some experimental conditions were therefore investigated to obtain optimal conditions for the following experiments.

Denatured VS non-denatured

Nagahara *et al.* (1998) emphasised the importance of denaturing TAT proteins before addition to cells. Therefore, TAT-IκBαWT was denatured with 8M urea and dialysed against cell culture medium. Figure 5-3-3-A shows the effect of denatured TAT-IκBαWT (30μg/ml) and non-denatured TAT-IκBαWT (30μg/ml) in HeLa cells. Both denatured (D) and non-denatured (ND) TAT-IκBα suppresses TNFα-induced luciferase activity, with the non-denatured protein showing the better suppression. Denaturing proteins may be required for larger proteins. However, in TAT-IκBα, because ND exerted better inhibition, the denaturing process was not chosen for the following experiments.

Preincubation time

In order to investigate the consequence of preincubation time, TAT-IκBα was incubated for 15 or 30 minutes before adding TNFα to the cells. According to Nagahara *et al.,* (1998), TAT enter T cells as early as 10 minutes. Using HeLa cells, 15 min or 30 min TAT-IκBα32,36 (30μg/ml) was preincubated before TNFα

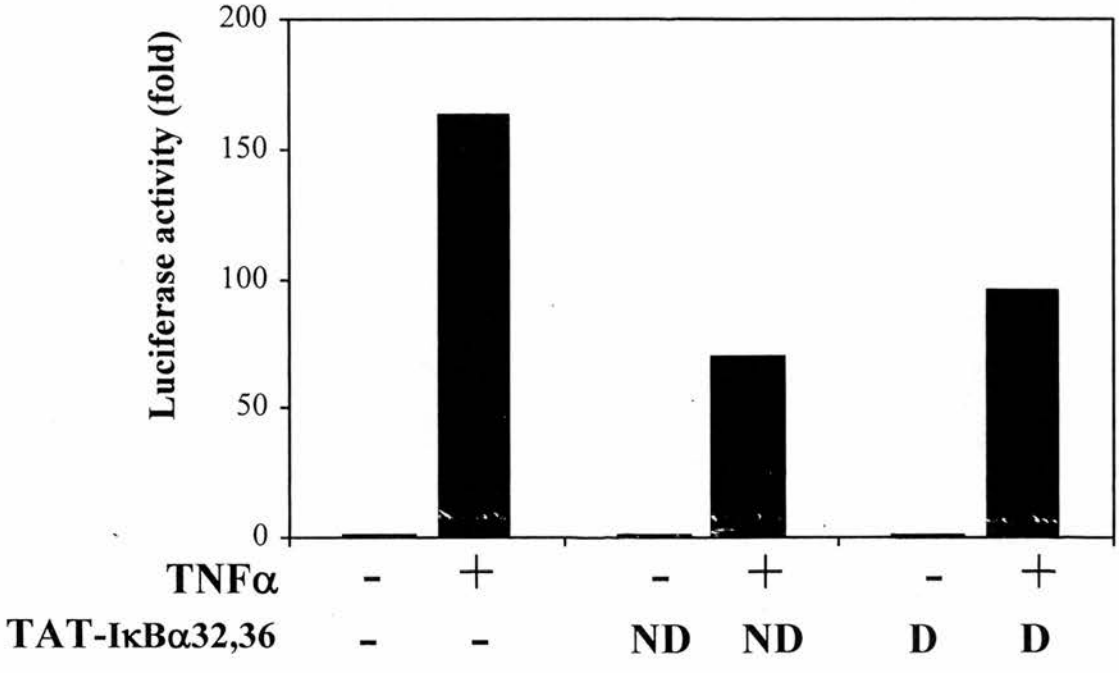


Figure 5-3-3-A; Denatured or non denatured TAT-IκBα WT inhibits TNFα induced NF-κB luciferase activity in HeLa cells.
HeLa cells containing NF-κB-dependent luciferase reporter gene was incubated with denatured **(D)** or non-denatured **(ND)** TAT-IκBα WT (30 μg/ml) for 30 minutes at 37°C and then treated with TNFα (5ng/ml) for 6 hours before luciferase expression was measured. The activation of luciferease was measured and was compared with untreated condition and expressed as fold (Y-axis). TAT-IκBα WT was denatured by 8M Urea. However, there was no advantage of denatured TAT-IκBα WT for NF-κB inhibition.

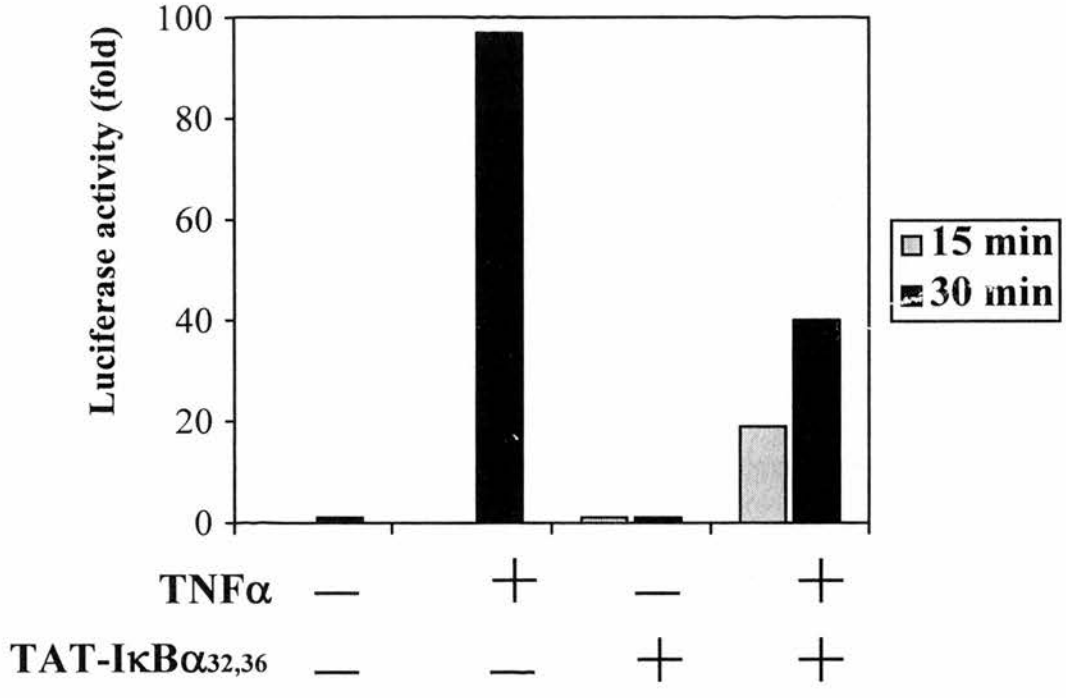


Figure 5-3-3-B; TAT-IκBα 32,36 inhibits TNFα induced NF-κB luciferase activity within 15 minutes of pre-incubation. HeLa cells containing NF-κB-dependent luciferase reporter gene was incubated with 30 μg/ml TAT- IκBα 32,36 (30μg/ml) for 15 minutes or 30 minutes at 37°C and then treated with TNFα (5ng/ml) for 6 hours before luciferase expression was measured. The activation of luciferease were measured and was compared with untreated condition and expressed as fold (Y-axis). A 15 minutes incubation was sufficient for inhibition of TNFα induced NF-κB luciferase activation.

(5ng/ml) was added. As shown in figure 5-3-3-B both preincubation times of 15 min or 30 min significantly suppressed TNFα-induced NF-κB activation. Moreover, a 15-min preincubation time exerts a slightly better inhibition than 30 minutes. Although 15 min pre-incubation period may be sufficient for suppressing NF-κB in HeLa cells, all the following experiments were performed using 30 min pre-incubation time for convenience (see 5.6.4. in discussion).

Washed and non-washed medium

Due to the nature of the luciferase experiment, reagents (e.g. TNFα, TAT-IκBα etc.) were left in the medium with the cells for 6 hours. To determine the effect of those reagents in the medium, cells were washed with fresh medium after treatment for 30 minutes with TAT-IκBα (30μg/ml) and 30 minutes with TNFα (5ng/ml) and incubated in fresh medium. After 6 hours incubation, luciferase activity was measured (data not shown). Generally washed cells exerted slightly lower luciferase activity. Thus, in order to obtain better luciferase activity, cells were incubated with the reagents for 6 hours rather than performing a washing step.

Serum Free Medium

Most experiments were performed with medium containing 10% FCS. However, in order to examine the effects of TAT-IκBα in serum free medium TAT-IκBα (WT) (30μg/ml) was co-incubated with TNFα (5ng/ml) in HeLa cells with the medium containing no serum. In serum free conditions, a similar dose-dependent inhibition of TNFα-induced-NF-κB was seen in serum containing medium (data not shown)

suggesting that the effect of TAT-IκBα was not dependent on the presence of serum. Following experiments were performed with medium containing 10% FCS.

Jurkat cells

Similarly to 57A HeLa cells, the Jurkat cell line, Jurkat α5.1 (Professor Hay, University of St Andrews), which contains luciferase reporter gene was used to examine the effect of TAT-IκBα. TAT-IκBα also inhibited TNFα-induced NF-κB activation in these cell lines (data not shown). However, the effect of TNFα itself on Jurkat α5.1 was limited compared to 57A HeLa cells (as shown previously). Jurkat cells were therefore not used for the further experiments.

Summary

The following conditions were selected for the following experiments;

- Non-denatured TAT-IκBα
- A 30 minutes incubation time with TAT-IκBα was used.
- Reagents (TAT-IκBα and TNFα) were kept with the cells and not washed away for luciferase expression
- Medium containing 10% FCS

5.4. TAT-IκBα32,36 in A549 cells

5.4.1. Western blotting analysis for IκBα in A549 cells

To investigate the effect of TAT-IκBα further, the epithelial cell line A549 was used for Western blotting (WB) and immunofluorescent microscopic analysis. TAT-IκBα32,36 (30 μg/ml) was incubated with A549 for 30 min at 37°C before TNFα

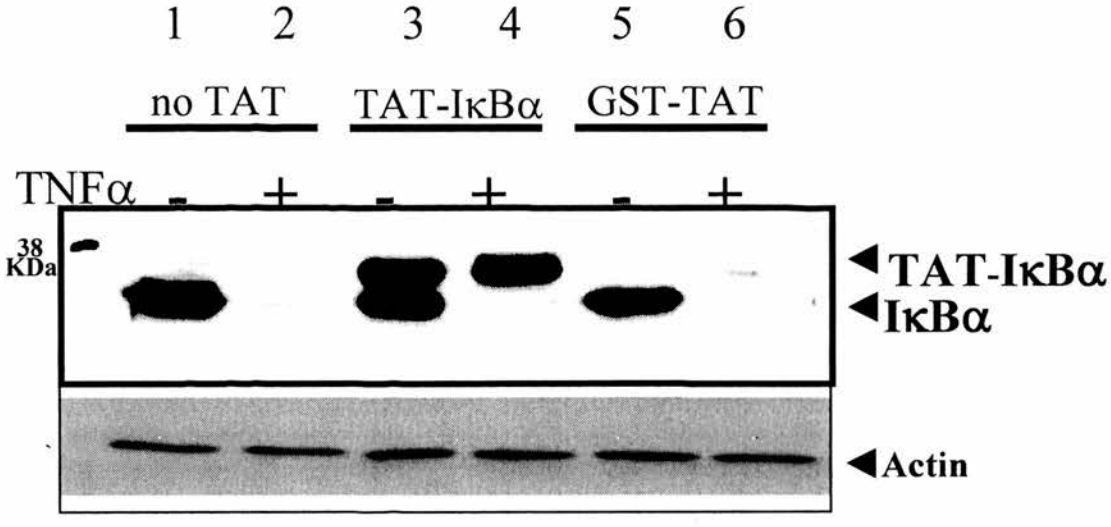


Figure 5-4-1; IκBα expression in A549 cells. A549 cells were incubated with TAT-IκBα 32,36 (indicated as TAT-IκBα) (30μg/ml), and GST-TAT (30μg/ml) for 30 minutes at 37°C and then treated with TNFα (5ng/ml) for 30 minutes. Cytoplasmic extract was analysed by Western blotting with polyclonal antibody specific for IκBα. Two forms of bands are seen. In the no TAT condition endogenous IκBα band (lower) is seen and degraded upon TNFα stimulation. Similar results are seen with GST-TAT. TAT-IκBα32,36 is seen as upper bands in lane 3 and 4 due to extra 11 amino acid TAT (see figure 5-2-1-4). TNFα stimulation degrades endogenous (lower) IκBα but keeps TAT-IκBα 32,36 (upper) remaining in the cells. This is a representative experiment out of four different experiments performed on the separate times.

(5ng/ml) was added and incubated for a further 30 min. Cells were lysed (see material and methods) and WB was performed using an anti-IκBα antibody to measure IκBα expression in the cytoplasm as an index of NF-κB activation. As shown in figure 5-4-1, two forms of IκBα are seen, a lower and an upper band. The conditions without any TAT (the 1st and 2nd lane from the left) shows endogenous IκBα (the 1st lane from the left) and its degradation by TNFα (the 2nd lane) as expected. Thus, the lower band is the endogenous normal form of IκBα. A similar pattern of IκBα degradation by TNFα is seen when GST-TAT (the 5th and 6th lane from the left) is used. Again, the lower band of IκBα (endogenous) is degraded by TNFα, when co-incubated with GST-TAT. Thus, in the presence of GST-TAT, TNFα likely releases NF-κB for the following transcription activities. However, with TAT-IκBα (lane 3 and 4), two forms of IκBα are seen. The lower band indicates an endogenous IκBα (see lane 1 and 5) and the upper band is TAT-IκBα32,36, which is 11 amino acids heavier than normal IκBα. Analysis by mass spectrometry of TAT-IκBα32,36 determined its molecular weight as 37570Da (see figure 5-2-4) whereas rIκBα is 35471Da. The upper band of IκBα indicated the presence of TAT-IκBα32,36, which transduced through the cell membrane into the cytoplasm. In lane 3 two forms of IκBα retain NF-κB in the cytoplasm, resulting in a strong inhibition of NF-κB. In lane 4 it is shown that the endogenous IκBα (lower band) is degraded, under TNFα stimulation, but clearly TAT-IκBα32,36 band (upper) does not get degraded, due to the mutations of serines 32 and 36. Although endogenous IκBα is degraded, over-expressed TAT-IκBα32,36 in the cytoplasm prevents NF-κB translocation and hence its transcriptional activity.

Summary

- Endogenous IκBα can be degraded by TNFα.
- TAT-IκBα32,36 transduces into the cytoplasm of A549 cells.
- TAT-IκBα32,36 is not degraded following TNFα stimulation.
- GST-TAT does not influence the degradation pattern of IκBα.

5.4.2. Immunofluorescence analysis of p65 in A549 cells

One of the common NF-κB dimers, p65 was examined in A549 cells and its translocation into nucleus upon NF-κB activation was monitored. TAT-IκBα32,36 (30 μg/ml) was incubated with A549 for 30 minutes at 37°C before TNFα (5ng/ml) was added and incubated for a further 30 minutes. Then cells were fixed and permeabilised before immunofluorescence staining with anti-p65 antibody. In figure 5-4-2 p65 is clearly seen in the cytoplasm in the untreated condition (top left panel). Upon TNFα stimulation, p65 is translocated into the nucleus as a result of the activation of NF-κB (top right panel). When using GST-TAT, the same pattern was observed following TNFα stimulation. Thus, GST-TAT does not influence TNFα-induced NF-κB activation. On the other hand, TAT-IκBα inhibits translocation of p65 into the nucleus causing p65 to remain in the cytoplasm with or without TNFα addition. Therefore, TNFα does not cause p65 translocation if TAT-IκBα32,36 is present in the cytoplasm. These results correlate with the Western blotting analysis of IκBα expression as shown in figure 5-4-1. The results with A549 cells confirms the results obtained with luciferase activation studied in HeLa cells as shown previously.

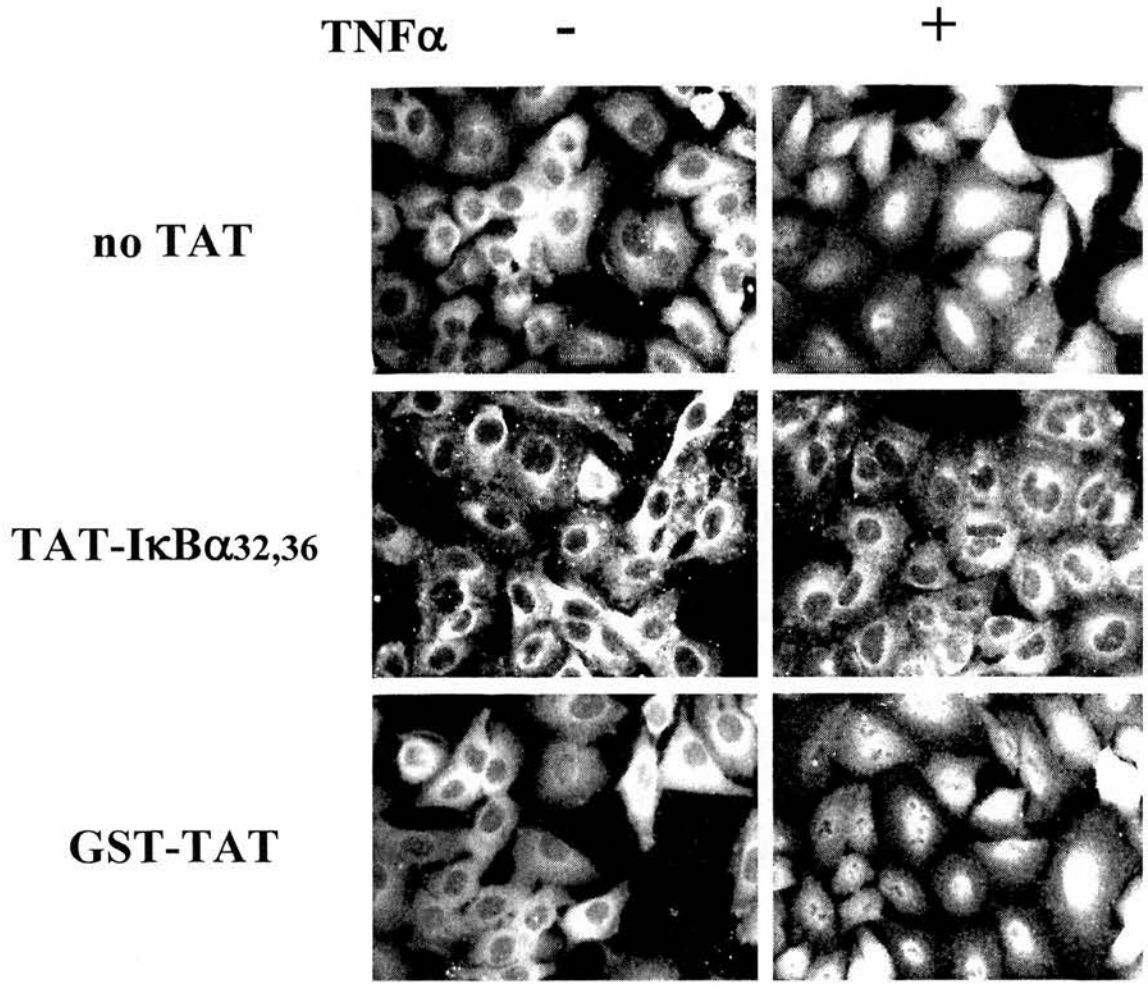


Figure 5-4-2; TAT-IκBα 32,36 inhibits TNFα induced p65 translocation in A549 cells. A549 cells were incubated with TAT-IκBα32,36 (30μg/ml) or GST-TAT (30μg/ml) for 30 minutes at 37°C and further treated with TNFα (5ng/ml) for 30 minutes. Cells were fixed and permeabilised for immunofluorescence staining with polyclonal anti-p65 antibody. In untreated cells p65 stays in the cytoplasm but translocates into the nucleus following TNFα stimulation. A similar pattern is observed in GST-TAT treated cells. However, TAT-IκBα32,36 inhibits p65 translocation by TNFα and retains p65 in the cytoplasm. This is a representative experiment out of three different experiments performed at separate times.

Summary

- TAT-IκBα prevents TNFα-induced-p65 translocation into the nucleus in A549 cells.
- GST-TAT does not prevent p65 translocation into the nucleus in A549 cells.

5.5. TAT-IκBα32,36 in eosinophils

5.5.1. TAT-IκBα transduces into eosinophils

Unlike established cell lines, eosinophils have almost no ability to be virally transfected. Thus, direct manipulation of intracellular proteins in eosinophils has never been demonstrated in the past. Here, TAT-IκBα is added to eosinophils to determine the possibility of transduction and inhibition of NF-κB.

Eosinophils were isolated as described in chapter 2. TAT-IκBα and recombinant IκBα (rIκBα) were conjugated with FITC for visualisation of internalisation of TAT into the cells. TAT-IκBα32,36-FITC (30μg/ml) and rIκBα-FITC (30μg/ml) were incubated with eosinophils for 30 minutes at 37°C in the dark. Cells were washed with PBS twice (5 minutes each) and cytocentrifuge preparations were obtained for observation under an immunofluorescent microscope as shown in figure 5-5-1. The cells with FITC positive are indicated by arrows. These FITC positive cells were observed in only TAT-IκBα32,36-FITC but not in rIκBα-FITC. These results suggest that TAT-IκBα32,36-FITC is transduced into eosinophils and that rIκBα-FITC is not.

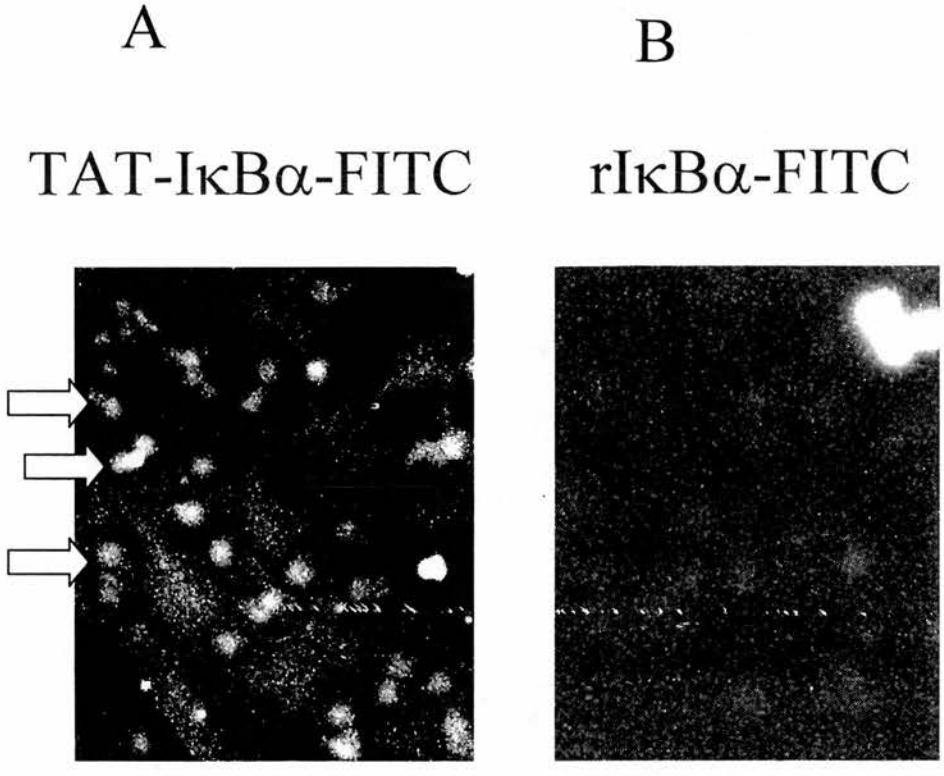


Figure 5-5-1; TAT-IκBα -FITC was transduced by eosinophil but not recombinant IκBα -FITC (rIκBα -FITC). FITC conjugated TAT-IκBα or rIκBα (both at 30μg/ml) were incubated with eosinophils for 30 minutes at 37°C in the dark. After incubation the cells were washed with PBS and cyto-centrifugated for immunofluorescent-microscope observation. The small white cells (arrows) are seen in panel A are FITC stained cells by TAT-IκBα-FITC, but not in panel B where rIκBα-FITC was not taken into the cells.

Summary

- TAT-IκBα32,36-FITC is transduced into eosinophils.
- rIκBα-FITC is not transduced into eosinophils.

5.5.2. IκBα and p65 expression by Western blotting in eosinophils

TAT-IκBα32,36 (30μg/ml) or GST-TAT (30μg/ml) were incubated with eosinophils for 30 minutes for 37°C then TNFα (10ng/ml) was added for a further 30 minutes before cells were lysed to obtain the cytoplasmic and nucleus extracts for Western blot (WB) analysis. WB was performed with anti-IκBα and -p65 antibody to examine the expression of IκBα in cytoplasm and p65 in nucleus as shown in figure 5-5-2.

In the cytoplasmic extract two bands are observed, as it has been shown in A549 cells (see figure 5-4-1); an endogenous IκBα (lower) and a TAT-IκBα (upper). Lane 1 (from the left) is the untreated control, where an endogenous IκBα is seen, while upon TNFα stimulation (lane 2) IκBα is degraded. A similar pattern was observed when GST-TAT was used (lane 5 and 6). Endogenous IκBα (lane 5, treated with GST-TAT) is seen as in the lane 1 for untreated control, but under TNFα stimulation (lane 6, treated with GST-TAT) IκBα is degraded with only a small amount of IκBα remaining in the cytoplasm. In lanes 3 and 4, when TAT-IκBα32,36 is used, the upper band of IκBα, TAT-IκBα32,36, is seen. When TAT-

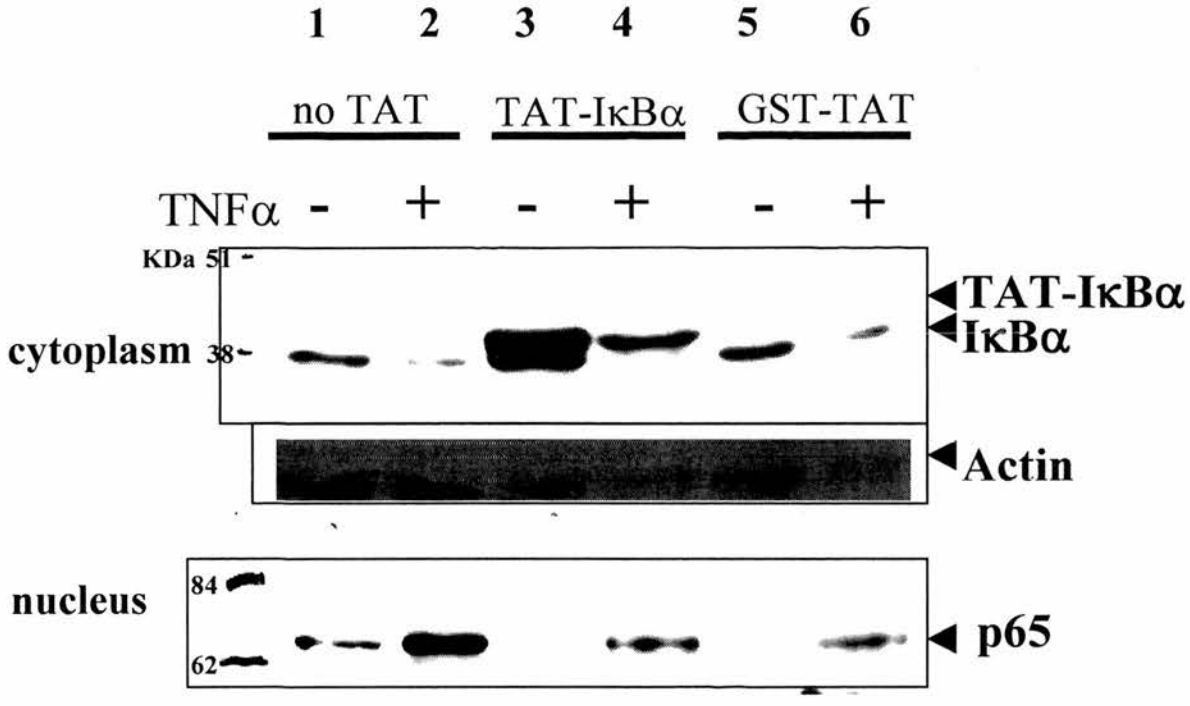


Figure 5-5-2; IκBα (cytoplasm) and p65 (nucleus) expression on Western blotting in eosinophils. Eosinophils were incubated with TAT-IκBα32,36 (indicated as TAT-IκBα) (30μg/ml) and GST-TAT (30μg/ml) for 30 minutes at 37°C and further treated with TNFα (5ng/ml) for 30 minutes. Cytoplasm and nuclear extracts were analysed by Western blotting using polyclonal antibodies specific for IκBα and p65. **Cytoplasm;** Two forms of bands are seen. In the no TAT condition endogenous IκBα band (lower) is seen but degraded upon TNFα stimulation. Same results are seen with GST-TAT pre-incubation. TAT-IκBα32,36 is seen as an upper band in lane 3 and 4 due to extra 11 amino acids TAT (see figure 5-2-3-1 for IκBα expression in A549 cells). TNFα stimulation degrades endogenous (lower) IκBα but keeps TAT-IκBα32,36 (upper) remaining in the cytoplasm. **Nucleus;** Clear p65 translocation is seen after TNFα stimulation in lane 2, compared with the untreated condition (lane 1). $3x10^6$ cells were used per condition.

IκBα32,36 is used, the presence of two forms of IκBα, TAT-IκBα32,36 are seen; an upper band (TAT-IκBα32,36) and an endogenous IκBα as lower band, in the cytoplasm indicating a strong inhibition of NF-κB. When stimulated with TNFα (lane 4), the endogenous IκBα (lower) is degraded. However, TAT-IκBα32,36 (upper) is resistant to degradation upon TNFα stimulation. These results are very similar to the results obtained using A549 cells (figure 5-4-1).

On the other hand, nuclear extract was examined with an anti-p65 antibody. Lane 2 shows TNFα induction of p65 in the nucleus is clearly seen compared to lane 1 without TNFα. With TAT-IκBα32,36 incubation (lane 3), no p65 appears indicating a strong NF-κB inhibition. In lane 4 a small amount of p65 is seen under TNFα stimulation when TAT-IκBα32,36 was incubated together. This is a smaller amount of p65 compared with TNFα only (lane 2). Thus, TAT-IκBα32,36 is indeed inhibiting TNFα-induced NF-κB activation in eosinophils. In lane 6 GST-TAT and TNFα showed slight p65 translocation. However, this is not as substantial as TNFα-induced p65 translocation as shown in lane 2.

Summary

- Endogenous IκBα is degraded when cells were stimulated by TNFα.
- TAT-IκBα32,36 transduced into the cytoplasm of eosinophils.
- TAT-IκBα32,36 is not degraded following TNFα stimulation.
- p65 is translocated into nucleus following TNFα stimulation in eosinophils.
- Translocation of p65 is prevented by TAT-IκBα32,36.

5.5.3. Eosinophil apoptosis

Inhibition of NF-κB induces apoptosis in many cells, especially when other death pathways, such as the caspases cascades are stimulated. In our previous study (see chapter 4, and Fujihara *et al,* 2002), when NF-κB inhibitors were used with TNFα in eosinophils, it is demonstrated that eosinophils undergo apoptosis.

Here, TAT-IκBα32,36 was used as a tool to investigate eosinophil apoptosis. Figure 5-5-3 shows eosinophil apoptosis (%), assessed by morphologically, when using TAT-IκBα32,36 (30μg/ml) and GST-TAT (30μg/ml) for 24 hours. In these experiments, untreated control and TNFα (10ng/ml) alone do not show significant differences between them. However, incubating eosinophils with TAT-IκBα32,36 dramatically induced apoptosis (>80%) with or without TNFα. This is a significant induction of apoptosis compared with the condition with no TAT-IκBα32,36 and GST-TAT. GST-TAT itself does not significantly induce apoptosis. There was a small induction of apoptosis when GST-TAT and TNFα were co-incubated. In conclusion, TAT-IκBα32,36 itself significantly induced eosinophil apoptosis. This effect appears to be further enhanced when TNFα was added. These results suggest that inhibition of NF-κB plays an important role in inducing eosinophil apoptosis. These results confirm the previous results shown in chapter 4.

However, it was notable that the induction of apoptosis was donor dependent. Some donors responded to TAT-IκBα32,36 significantly to induce apoptosis as shown in

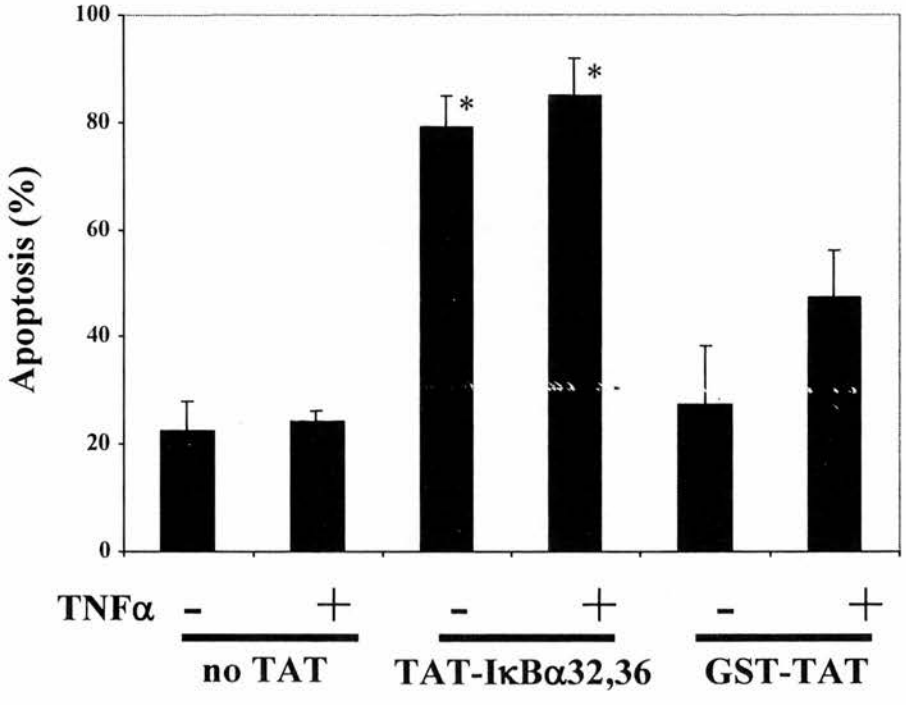


Figure 5-5-3; Eosinophil apoptosis induced by TAT-IκBα32,36.
Human eosinophils (3 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum with TAT-IκBα32,36 (30μg/ml) and GST-TAT (30μg/ml) for 30 minutes at 37°C and further treated with TNFα (5ng/ml) in the indicated conditions. At 24 hours, cells were cytocentrifugated for microscopic observation and apoptotic cells were assessed by %. TAT-IκBα32,36 induced eosinophil apoptosis significantly. All values represent mean ± S. E. of n = 3 experiments. * represents significant difference from appropriate controls ($p<0.05$).

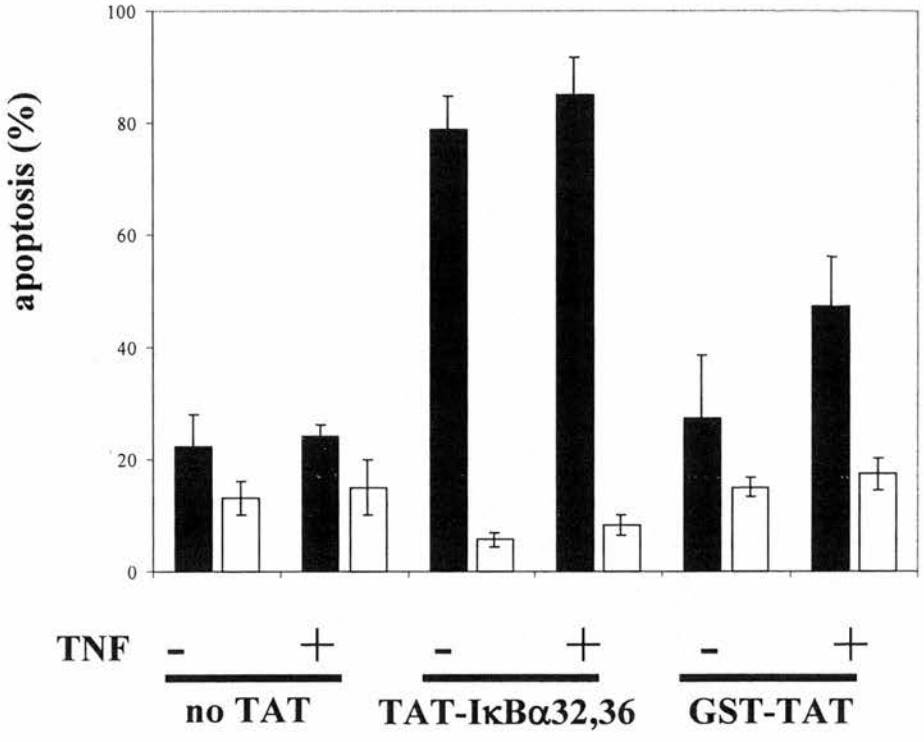


Figure 5-5-3-b; Eosinophil apoptosis induced by TAT-IκBα32,36 in two types of groups; responded- and non-responded.

Human eosinophils (3 x 10^6/ml) cultured in Iscove's DMEM containing 10% autologous serum with TAT-IκBα32,36 (30μg/ml) and GST-TAT (30μg/ml) for 30 minutes at 37°C and further treated with TNFα (5ng/ml) in the indicated conditions. At 24 hours, cells were cytocentrifugated for microscopic observation and apoptotic cells were assessed by %. TAT-IκBα32,36 induced eosinophil apoptosis significantly. All values represent mean ± S. E. of n = 3 experiments. * represents significant difference from appropriate controls ($p<0.05$). There are two types of group of subjects; responded (filled bar) and non-responded (open bar) to TAT-IκBα32,36-induced apoptosis.

figure 5-5-3, while some populations did not (Figure 5-5-3-B). The difference between those populations in terms of induction of apoptosis is not known. However, it is likely that eosinophils from those donors may have activated pathways, such as NF-κB or tyrosine cascades, that may also interfere with the apoptotic pathway.

Summary

- TAT-IκBα32,36 itself induces eosinophil apoptosis.
- These results suggest the importance of inhibition of NF-κB.
- GST-TAT and TNFα induce eosinophil apoptosis to a small extent.
- Eosinophils from some donors did not respond to TAT-IκBα32,36.

5.6. Discussion

5.6.1. TAT as a tool for internalisation.

Direct internalisation of a target peptide, protein, enzyme, etc. using TAT into various types of cells has widened the potential of a direct approach to manipulate target intracellular proteins. Here, it has been shown that TAT-IκBα is successfully transduced into HeLa cells, A549 cells and eosinophils. Although not shown here, TAT-IκBα-FITC also confirmed successful internalisation into neutrophils. Granulocytes are thought to be extremely difficult cells to use any direct approaches to manipulate intracellular pathways, such as viral transfections due to lack of specific receptors and their short lives. However, this TAT peptide is a promising method to transduce protein into granulocytes.

5.6.2. TAT-IκBα

It has recently been shown that internalisation of TAT-IκBα in different cell types inhibits activation of NF-κB (Kabouridis *et al.*, 2002). In HeLa cells luciferase activity clearly demonstrated that the inhibition of NF-κB, especially TNFα-induced NF-κB activation (figure 5-3-2) by TAT-IκBα. In A549 cells, internalisation of TAT-IκBα in the cytoplasm was shown by Western blotting (figure 5-4-1). Over-expressed IκBα in the cytoplasm prevents translocation of p65 into nucleus. Furthermore, p65 translocation was examined by immunofluorescence (figure 5-4-2). It has been demonstrated that TAT-IκBα indeed prevents the translocation of p65 into the nucleus despite stimulation of TNFα at the same time.

These data obtained from HeLa and A549 cells strongly suggest that TAT-IκBα inhibits NF-κB.

5.6.3. Optimal conditions for the usage of TAT-IκBα

Because the usage of TAT was a new technique in not only eosinophils but also cell lines, optimal conditions had to be investigated for our experiments. Various conditions were tested in HeLa cells then those conditions were applied to eosinophils and A549 cells.

In this study, as the control protein for TAT-IκBα, GST-TAT was chosen. This is due to the process of cloning (see figure 5-2-1), GST-TAT was thought to be the optimal control for this study. Similarly, other groups such as Kaburidis *et al.*, (2002) have also used GST-TAT as a control for TAT-IκBα. Ideally, TAT-IκBα which is functionally unable to inhibit NF-κB would be the best control. For instance, TAT-IκBα which is mutated at NF-κB binding sites and therefore incapable of holding NF-κB in the cytoplasm would be the best control protein. This study should be performed for the next step.

Previously, TAT-p27 (Nagahara *et al.*, 1998) and TAT-H-Ras (Hall *et al,* 2001) were shown to start internalising into cells as early as 5 minutes. In HeLa cells as it has been shown in figure 5-3-3-B, a pre-incubation time of 15 min with TAT-IκBα was sufficient. However, in this study 30 min incubation time was used. In our previous study in chapter 4, gliotoxin and Mg132 were pre-incubated for 30 min

before TNFα was added. In order to have consistent experiments from the previous studies, 30 min pre-incubation of TAT-IκBα was chosen.

After the internalisation of TAT, its actual movement within the cells is not known. It is a possibility that TAT-IκBα may not only go through cell membrane but also may enter the nucleus. It has been demonstrated that endogenous IκBα goes through the nuclear membrane (Arenzana-Seisdedos *et al.*, 1995) and transports NF-κB back to the cytoplasm. Therefore, it is possible for TAT-IκBα to go into the nucleus and export NF-κB from the nucleus back into the cytoplasm. Unfortunately, this point was not investigated during this project. However, it would be interesting to examine; 1. IκBα expression in nucleus extracts by Western blotting 2. IκBα expression by Immunofluorescence or confocal microscope study. In addition, the function of TAT-IκBα can be investigated with immunoprecipitation to demonstrate if p65 or p50 is actually held by TAT-IκBα. These experiments would answer the more specific questions of the function of TAT-IκBα in cells.

5.6.4. Denatured and non-denatured TAT-IκBα

Although Nagahara *et al.,* (1998) emphasised the importance of denaturing proteins, most of the reported papers had used denatured TAT-protein. However, it appeared that this process is not necessary for TAT-IκBα. The requirement for denaturing process can be dependent upon the shape and size of the protein, where some proteins were easy to get into cells whereas some were not. For some reason IκBα may have the shape or other natures that make TAT-IκBα transduce easily

into cells. However, in the same Dowdy's group, it is mentioned that 40nm super paramagnetic beads were successfully transduced into T cells, which is no bigger than 4 μm (Schwarze *et al.*, 2000). This evidence raises the question for the requirement of denaturing process if such a size of the beads can enter cells. It may be due to the smooth surface of the beads that helped them into cells easily. Therefore, odd shaped proteins might need the denaturing process although their actual size is smaller than 40nm beads. These studies to investigate the shape and composition of the protein may help understanding the mechanism of the TAT internalisation into cells. Furthermore, it could be possible in the future to design the protein linked with TAT that selectively target internalisation into specific cell types for the therapeutic purposes.

5.6.5. Apoptosis in eosinophils

From studies of HeLa and A549 cells, there is strong evidence showing that TAT-IκBα inhibits NF-κB activation. In eosinophils, Western blotting analysis (figure 5-5-2) demonstrates that over-expression of IκBα in the cytoplasm results in the inhibition of translocation of p65 into the nucleus. This is the first demonstration that TAT-IκBα has been successfully transduced into eosinophils and shown to functionally inhibit NF-κB both with or without TNFα stimulation.

As it has been shown in figure 5-5-3, TAT-IκBα induces eosinophils apoptosis. Interestingly, this implicates that TAT-IκBα does not require TNFα cytotoxity but can induce eosinophil apoptosis by itself. This outcome is more dramatic than our previous results shown in chapter 4 using gliotoxin and Mg132. It may be that TAT-

IκBα itself was toxic to the cells. The concentration might not be optimal. However, as has mentioned in 5.5.3., this effect was donor dependent, in other words, in some donors TAT-IκBα did not exert its effect to induce apoptosis at all. Therefore, concentration may not be the issue for the cytotoxity to eosinophils.

TAT-IκBα32,36 blocks signal induced activation of NF-κB dependent reporter gene activation in HeLa cells. In A549 cells TAT-IκBα32,36 does not block TNFα induced degradation of endogenous IκBα but appears to bind the released NF-κB and retain it in the cytoplasm. This is consistent with recently demonstrated works in HeLa cells. Uptake of TAT-IκBα 32,36 into eosinophils induces apoptosis in the presence or absence of TNFα. This indicates that a basal level of NF-κB is essential for eosinophil survival in culture, presumably to content a constitutively activated pathway. The ability of IκBα to translocate to the cell nucleus and export NF-κB complexes back to the cytoplasm would suggest that TAT-IκBα32,36 could inhibit basal NF-κB dependent transcription activities, which influence apoptosis. Although the basics for the heterogeneity of this response is unknown, it is likely to be related to the extent of eosinophil activation and consequent NF-κB translocation in these proteins.

Indeed, there could be two distinct populations that TAT-IκBα exert high sensitivity or no effect at all especially for inducing apoptosis. To investigate this point, ideally, dose response of TAT-IκBα may be required for each donor. However, to perform this kind of experiment, large number of eosinophils are required. Therefore, this would be very difficult series of experiments to be performed. In addition, other methods (e.g., FACS analisys of Annexin V or DNA ladder etc.)

should be employed to confirm apoptosis rather than morphologically. Since this result which is donor dependent was totally unexpected outcome. Therefore, we did not have any symptoms scores etc. to correlate. However, this is the next project to determine the correlation between response to TAT-IκBα and other factors, as such, allergic symptoms scores, if asthmatics FEV1, PC20, skin prick tests, peripheral eosinophil counts, peripheral level of IgE.

There is no clear evidence or reason for the outcome of two different populations in terms of the apoptosis assay. However, in my opinion, the difference may be based on the state of the activation of eosinophils. Towards the end of this project, my guess was that donors who did not respond to TAT-IκBα tended to be the 'high eosinophil donors' who may likely to have allergic diseases, such as eczema and asthma. In these donors, eosinophils are likely to be 'activated' when they were isolated from peripheral blood. Unfortunately, there is no direct evidence to support this hypothesis. Therefore, for the next step, it would be very interesting to investigate this possibility, which can be examined by eosinophil counts in peripheral blood, eosinophil activation by flow cytometry, activation level of other signalling pathways and the production of pro-inflammatory cytokines.

There could be a few reasons why TAT-IκBα does not induce apoptosis in some populations. In eosinophils IL-5 triggers Jak-STAT 1 (Paxdrak *et al.*, 1995), Lyn, Syk (Yousefi *et al.*, 1996) Jak 2 (Paxdrak *et al.*, 1998) pathways, which have been shown to be the crucial eosinophil survival pathways. Thus, in some donors, tyrosine kinase cascades might be more important for eosinophil survival than NF-κB dependent pathways. This could be investigated by blocking those tyrosine

pathways in the same time using TAT-IκBα. In this project, the study of tyrosine pathways was not included since NF-κB was the primary aim. However, to gain a clear answer, to examination of tyrosine pathways should be included in eosinophils apoptosis study. Because intracellular pathways are complex involving many pathways, proteins, organelles etc. many as yet unknown pathways may exist to control eosinophil apoptosis. The answer to their interesting questions will have to await for future studies.

Chapter 6
Hodgkin's Disease and eosinophilia

6.1. Introduction

6.1.1. Hodgkin's disease

The first description of Hodgkin's Disease (HD) made by Thomas Hodgkin in 1832 at Medical School in Edinburgh University was 'some morbid appearances of the absorbant glands and spleen in lymph nodes' (Hodgkin, 1832). Almost 60 years after Hodgkin's observations further characterisation of the disease was made by Dorothy Reed and Carl Sternberg. They described the manifestation of unusual large mononuclear cells which were named as Hodgkin (H) cells making up less than 1% of the total tumour together with Reed-Sternberg (RS) cells which in contrast are multinucleated (Reed, 1902, Sternberg, 1898, reviewed by Küppers and Rajewsky, 1998).

Hodgkin's disease (HD) or Hodgkin's lymphoma is the most common lymphoid malignancy in the western world, affecting mainly young adults and people older than the age of 55 years of age. The main symptoms of Hodgkin's disease are painless swelling of the lymph nodes in the neck, underarm, or groin, fever, night sweats, tiredness, weight loss, itchy skin, and there may be increased susceptibility to infection due to an imbalance of the immune system. Accurate determination of the progress and categorisation of Hodgkin's disease is by microscopic observation of biopsy samples following surgery (see figure 6-1-1 for biopsy sample). Further diagnosis is then determined with appropriate treatment.

The most standard treatment for HD is the combination chemotherapy MOPP (Mechlorethamine, Vincristine, Procarbazine, Prednisone) and ABVD (Doxorubicin, Blemycin, Vinblastine, Dacarbazine), which both demonstrate the

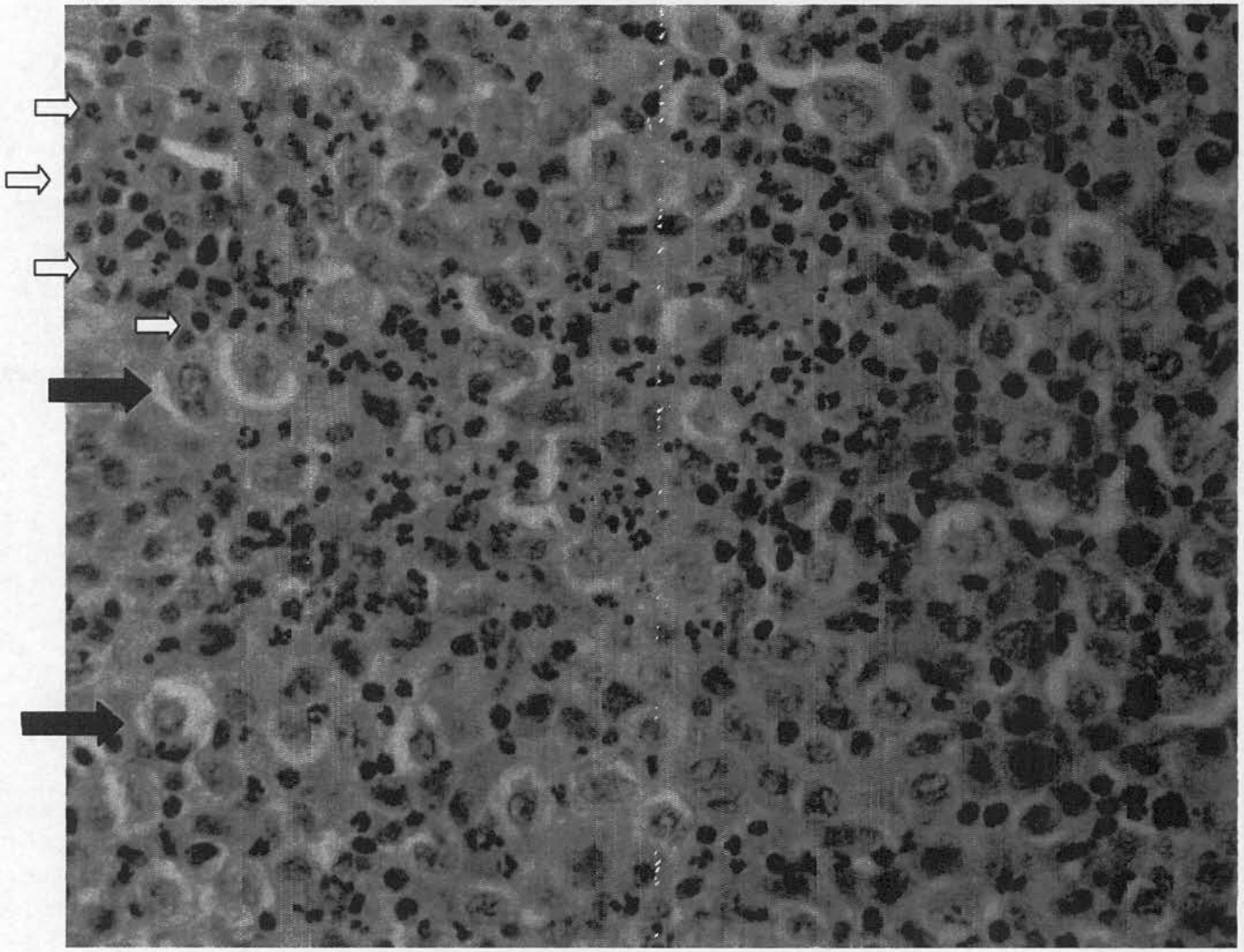


Figure 6-1-1-A; Biopsy materials of HD with eosinophilia. The biopsy sample of HD with eosinophilia Biopsy material was taken from lymph node from the patients. This sample contains abundant amounts of eosinophils (white arrows) and Hodgkin-Reed-Sternberg (HRS) cells (black arrow) are seen in the tissue . This photo was kindly provided by Prof Ruth Jarrett, University of Glasgow.

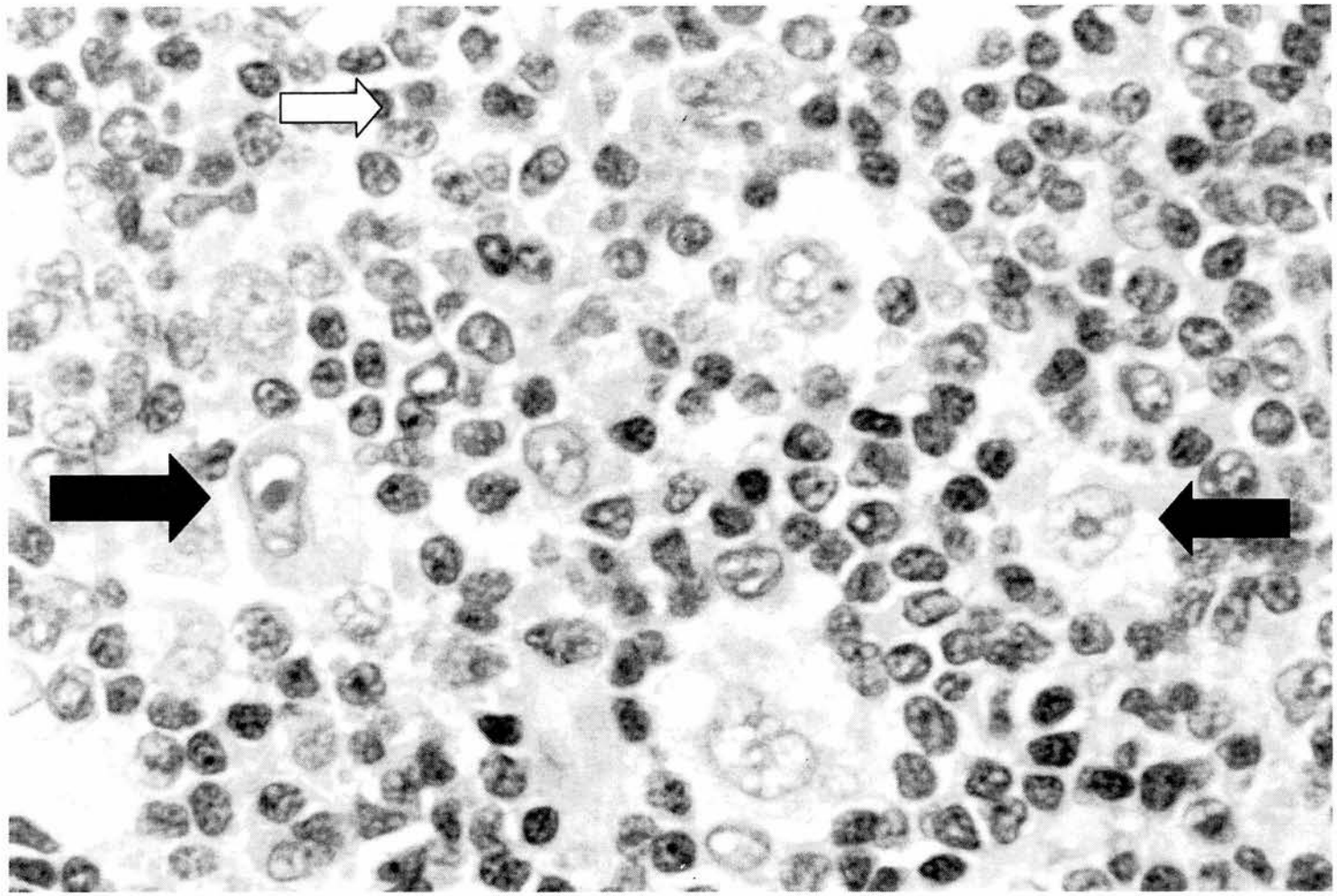


Figure 6-1-1-B; Biopsy materials of HD with eosinophilia with typical Hodgkin-Reed-Sternberg (HRS) cells. HRS cells (black arrow) with typcial 'owl eyes' and eosinophils (white arrow) are seen. Biopsy material was taken from lymph node from the patients. This photo was kindly provided by Prof Ruth Jarrett, University of Glasgow.

superior effects. In addition to the chemotherapy, patients may receive radiotherapy for advanced tumour.

HD can be categorised into four subgroups according the composition of the cells, for example, Lymphocyte predominant (LP), lymphocyte-depleted (LD), nodular sclerosis (NS) and mixed cellularity (MC) (reviewed by Burke *et al.*, 1992).

6.1.2. HRS cells and inflammatory cells

Although being defined by the presence of cancerous Hodgkin Reed-Sternberg (HRS) cells, more than 98% of the tumour in Hodgkin's disease is composed of non-cancerous cells. The accumulated inflammatory infiltrate consists predominantly of eosinophils and T cells, with less notable abundance of neutrophils, basophils, macrophages and B cells. As shown in figure 6-1-1-A and B eosinophils are commonly observed in the biopsy samples of HD. These features of HD therefore share some similarities to the sites of inflammation.

Although the notable number of eosinophils and other inflammatory cells at HRS cells has been known for many years, the function and mechanism of the accumulation of inflammatory cells, are not fully understood. Previously eosinophils were thought to be a 'bystander' and 'innocent' cells occurring during the disease process. However, recently the role of the eosinophil has come into question in light of clinical reports suggesting that the eosinophilia at HRS cells correlates to the poor prognosis in patients (Enbald *et al.*,1993, von Wasieleski *et al.*, 2000). Enbald *et al.* (1993) and von Waseileski *et al.* (2000) have reported that patients with eosinophilia at the tumour in comparison with ones with non-eosinophilia had

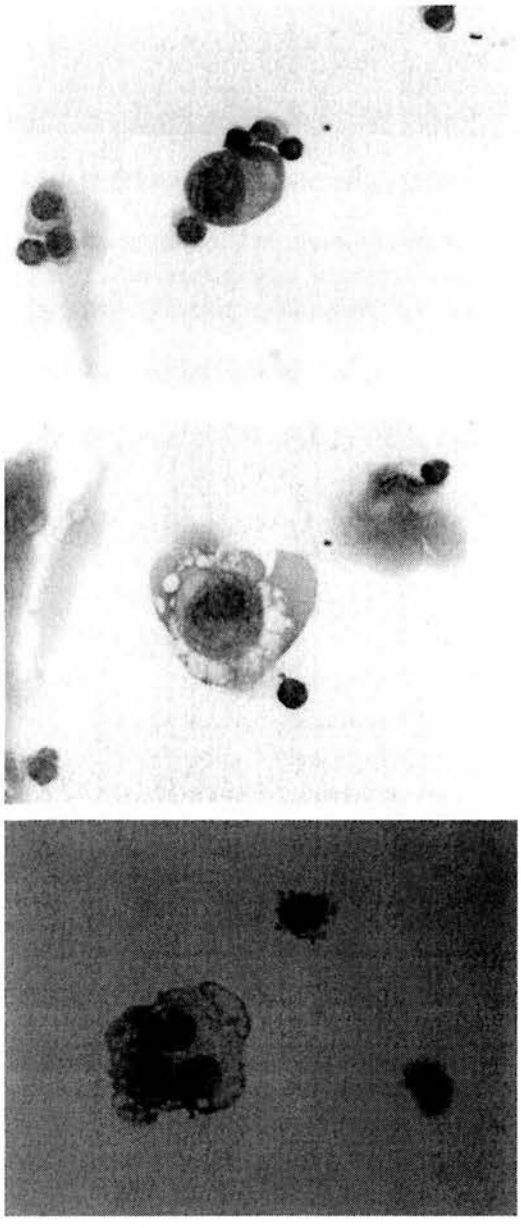


Figure 6-1-2; Isolated HRS cells from biopsy samples. HRS cells were purified and isolated from biopsy materials. These photos were kindly provided by Professor R Jarrett, University of Glasgow.(Magnification x 100)

significantly poor prognosis for survival. Recently, von Wasielewski *et al.* (2000) have suggested a possible negative influence of eosinophils in Hodgkin's disease, which may be regulated by constitutive activation of NF-κB. (Also see introduction of chapter 1, 4 and 5 for a description of NF-κB activation as pro-inflammatory in eosinophils.) It appears that eosinophilia may potentially exert a deleterious effect to the host, but, currently the reasons and role of eosinophils in HD are not known.

T cells are another cell type commonly observed in HRS tumour. It has been demonstrated that T cells rosette spontaneously to HRS cells assessed both *in vitro* and *in vivo* (Sanders *et al.*, 1988). There is some evidence suggesting that T cells at HRS cells express CD4+ (Poppema *et al.*, 1998), CD30L and CD40L (Pinto *et al.*, 1997) and that T cell activation may lead to tumour progression, rather than cytotoxic anti-tumour properties (Poppema *et al.*, 2000). Recent reports have suggested that the interaction between T cells, eosinophils and HRS cells may contribute to cellular activation of HRS cells. Expression of CD30L, CD40L, CD40, CD95/Fas and CD95/FasL on eosinophil surface appears to be higher in hypereeosinophilic syndrome (HES) patients in HD than in normal healthy donors (Pinto *et al.*, 1996 and 1997). CD30 was originally identified as a surface antigen on Reed-Stenberg cells and found to be preferentially expressed on activated CD4+ T cells (Del Prete *et al.*, 1995, Manetti *et al.*, 1994). Interestingly, CD30 ligand binds to CD30 on HRS cells and promotes cell proliferation, cell survival and production of IL-3, IL-5 and GM-CSF (Elliss *et al.*, 1993, Alzona *et al.*,1994, Del Prete *et al.*,1995, Mosmann *et al.*, 1996). These products also suppress eosinophil apoptosis. Therefore, interaction between T cells and HRS cells may promote activation of not only HRS cells but also other inflammatory cells, especially eosinophils.

Other types of cells may also contribute directly or indirectly to the progress of HD. For example, in the surrounding tissue, release of eotaxin from fibroblasts may contribute to the infiltration of eosinophils (Jundt *et al.*, 1999). Furthermore neutrophils may also have a part to play in tumour formation.

It appears to be that the host immune mechanism by some unknown reason is triggered by HRS cells resulting in the infiltration of normal inflammatory cells at the site of HRS cells. Accumulation of inflammatory cells may directly contribute to the formation of the tumour. A greater understanding of the nature of HD may be obtained by gaining further insight into the mechanisms underlying the accumulation of inflammatory cells in HD.

6.1.3. Activation of HRS cells

Similarly to other cancerous cells, HRS cells posses distinct features; such as high cellular activation and enhanced survival, possibly due to disruption of normal cell activities, often caused by the abnormal action of cellular signalling pathways. There is significant evidence that the cause of high cellular activation in HRS cells may be due to the constitutive high activation of NF-κB (Cabanne *et al.*, 1999 and Krappmann *et al.*, 1999), the absence of caspase-3 expression (Izban *et al.*, 1999), deficiency of CD95-induced apoptosis (Re *et al.* 2000, Muschen *et al.* 2000) and infection with Epstein-Barr Virus (EBV). For example, Cabanne *et al.* (1999) and Krappmann *et al.* (1999) have shown that the mutated deletion and abnormal function of IκBα in the cytoplasm in HRS cells leads to abnormally high activation of NF-κB, which act in an anti-apoptotic manner and may be involved in the

production of cytokines and chemokines, promoting the recruitment of inflammatory cells. For example, the IL-8 gene is often upregulated by NF-κB activation. (See chapter 4.) Thus, abnormal NF-κB activation in HRS cells can cause infiltration of inflammatory cells and result in progression of disease.

EBV can infect human B cells and be commonly involved in various haematological diseases (e.g. African Burkitt's lymphoma, post-transplantation B cell lymphomas and certain T cell lymphoma) including HD (Weiss *et al.*, 1996). The role of EBV in the pathogenesis of HD is not fully understood. However, recent investigations suggest that EBV may be one of the causes for the mutations within the cells to result in the formation of HRS malignant cells from normal haemopoetic cells (Küppers and Rajewsky, 1998). Furthermore, EBV positive HRS cells have higher cellular metabolism and activation, including NF-κB activation, resulting in an increased production of various cytokines such as IL-10, (Vockerodt *et al.*, 2001) and elevated expression of surface receptors such as CD25 (Kube *et al.*, 1999). Thus, EBV may play an important role in the progress of HD. In the HRS cell lines, L428, L540, KMH2 (Vockerodt *et al.*, 2001) are positively infected with EBV.

6.1.4. Origin of HRS cells

From microscopic observation, it appears that the morphology of HRS cells is quite unique, containing 'ovoid' nuclei, and differs from the characteristic identifying nature of other haematopeotic cells. Figure 6-1-1-B shows typical HRS cells containing ovoid nuclei. Figure 6-1-2 shows the isolated HRS cells from biopsy samples containing one to three nuclei per cell. There have been numerous studies investigating the origin of HRS cells. However, often technical difficulties prevent

HRS cell lines	L540	L428	L591	KMH2	HDLM2	HDMYZ
Histological subtype	nodular sclerosis	nodular sclerosis	nodular sclerosis	mixed cellularity	nodular sclerosis	nodular sclerosis
Origin of material	peripheral	pleural effusion	pleural effusion	pleural effusion	pleural effusion	pleural effusion
Age/ Sex	20/F	37/F	31/F	37/M	74/M	29
Clinical stage	IV-B	IV-B	IV-B	IV	IV	
Cell kinetics	Lymphoid (L)	L	L	L	L	
Immuno-phenotypes/ genotype	T cell (T)	incl*	B cell (B)	B	T	Macro-phage
Functional studies	L	L, Dendritic cells	L, B	L, Histocyte (H)	L, T, H	
Chromosome no.	66-71	48-50	66-71	>44	36	
NBT reduction phagocytosis	-	+/-	-	-		
Neutrophil-Migration-Inhibitor	+	+				
EVB	+	+/-	+	+/-	-	-
IκBα*	deletion 30KDa	WT	WT	deletion 18KDa	WT	WT
Spontaneous T cell rosette	+/-	+	+			

Table 6-1-4; Summary of HRS cell lines establishment.
(Modified from Drexler 1993, HDMYZ data from Bourour *et al,* 1993)
The nature, origin and the original patients' information of six different well established cell lines are shown. Typically, most of the cell lines have been established from patients, younger than 40 years at the end-stage of disease. The 'origin' of the most cell lines are lymphoid, T cells or B cells, except HDMYZ. T cell rosettes have been reported in L540, L428 and L591, but eosinophil rosettes have never been reported in any cell lines. 'incl' indicates inconclusive. NBT is nitroblue tetrazodium used to indicate uptake in a phagocytosis assay. * IκBα study by Cabanne *et al.*, 1999.

accurate determination of the nature and origin of HRS cells. The problems of purification of HRS cells are due to the large number of contaminating inflammatory cells and small number of HRS cells obtained following purification. Furthermore, often HRS cells do not survive long enough for establishing cell line cultures.

However, despite the technical difficulties, successful study of HRS cells by surface marker expression by PCR and DNA analysis (e.g. southern blotting) have demonstrated their origin as T cells (TCR expression, CD2, CD3, CD4 etc.) or B cells (Ig heavy and light chains, CD20, CD75 etc.). Table 6-1-4 summarises the origin of HRS cell lines, for example, gene rearrangement studies demonstrate that L540 and HDLM2 have TCR gene arrangement, whereas L591 and KMH2 have Ig gene rearrangement and L428 has both. In clinical samples, about 80% of HRS cells are B cell in origin from LP and about 30% of them are T cell positive in LD, NS and MC. Furthermore a recent study demonstrated that HRS cells share a similar phenotype and function as dendritic cells (Kadin, 1994). In normal situations B cells, T cells and dendritic cells have diverse functions from each other yet as a consequence of becoming HRS cells, they all exert similar functions, for example infiltration of inflammatory cells. The ability of diverse cell types to perform similar function upon becoming HRS cells requires further investigation.

6.1.5. Establishment of HRS cell lines

There are relatively few HRS cell lines in comparison with other cancer cell lines due to difficulty in establishing HRS cell lines. Table 6-1-4 summarises the origin of the cells from patients. For example, Diehl and co-workers have established

L428, L591 and KMH2 cell lines from bone marrow or pleural fluids taken from patients with end-stage of HD (Diehl *et al,* 1981). For unknown reasons, there is a better chance of establishing cell lines from patients with progressed malignancy (Professor R Jarrett, University of Glasgow, personal communication). Thus, HRS cells from the more aggressive tumours may be an essential factor for establishing successful cell lines.

6.1.6. Substance x; chemoattractant and survival factors for eosinophils

The mechanism of infiltration and accumulation of inflammatory cells at HRS cells may be due to release of various chemoattractants from HRS cells, surrounding cells in the tissue, such as fibroblasts, and inflammatory cells themselves once at the site of HRS cells (see figure 6-1-6 for a diagram). Furthermore, following the recruitment of those inflammatory cells, there may be some substances released resulting in high cellular activation and enhanced survival of inflammatory cells. As a consequence activated inflammatory cells are able to successfully escape from the normal induction of apoptosis which results in clearance of dying cells by macrophage phagocytes. In this thesis, this substance(s) is called substance x, which causes eosinophil chemoattraction, activation and survival.

The possibility exists that substance x can be either a known or unknown peptide, protein or lipid or a cocktail of these substances. The list of candidate substances seems endless and may even vary in different cell lines and biopsy materials.

However, in this thesis, in an attempt to understand the mechanisms responsible for eosinophil accumulation at HRS cells, the survival effect of eosinophils by soluble factors in the supernatant from HRS cells lines were investigated.

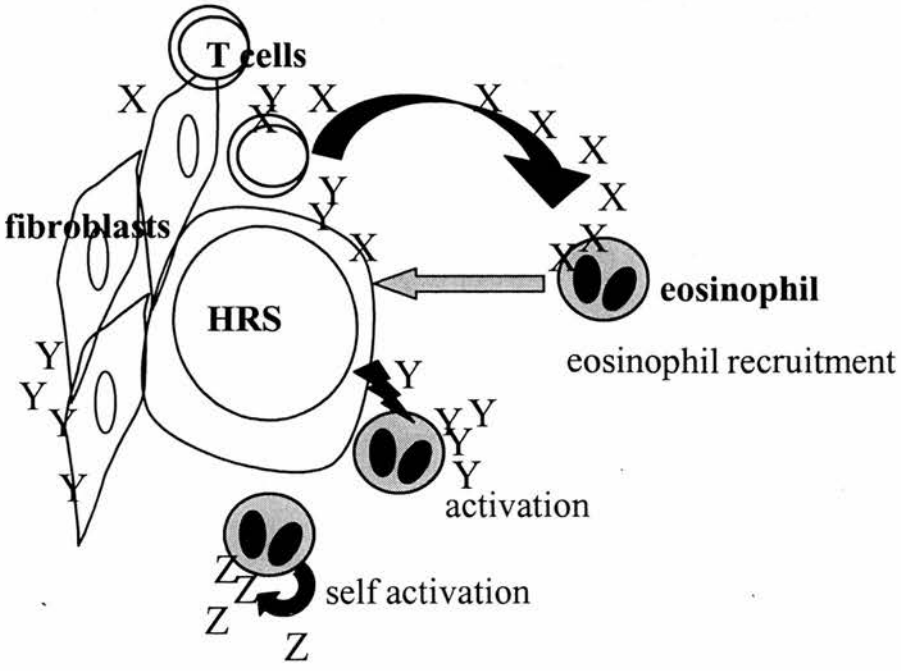


Figure 6-1-6; The diagram summarises possible causes of eosinophilia at HRS cell tumours. Eosinophil chemoattractants (indicated as X), activators or survival factors (Y) may be released by HRS cells, T cells and surrounding tissue, such as fibroblasts. Also, activators and survival factors (Z) may be released from the eosinophils themselves resulting in positive feedback activation and survival.

6.1.7. Possible candidates for substance x

HRS cell lines and biopsy samples have been studied by gene expression and by measuring the generation of substances, for example cytokines, chemokines and adhesion receptors all of which may influence inflammatory cells recruitment, activation and survival. Despite these previous studies, there has been no clear answers explaining the accumulation of inflammatory cells in HRS tumours. An investigation of the causes and mechanism of the infiltration of inflammatory cells, especially of eosinophil is investigated in this thesis. It is already known that some cytokines and chemokines are known to cause eosinophilia. Table 6-1-7-A summarise the candidates cytokines and chemokines for substance x and are listed according to their molecular weight. They are therefore possible candidates for eosinophil accumulation at HRS cells. A literature summary of eosinophilic cytokines and chemokines possibly present and produced by HRS cell lines and biopsy samples are shown in table 6-1-7-B. Cytokines and chemokines are measured by various methods (e.g. protein level, mRNA, microarray, southern blotting etc.) in various HRS cell lines and clinical samples.

Among various cytokines and chemokines as shown in table 6-1-7-B, some chemokines, such as eotaxin, IL-8, TRAC, RANTES, and cytokines, such as TNFα, TGFβ, IL-2, IL-3, IL-5, GM-CSF and IL-13 modulate various aspects of eosinophil function. Among them are eosinophil chemoattractants such as IL-2, RANTES, IL-8 and eotaxin. Eosinophil activators are most of the chemokines and cytokines such as IL-3, IL-5, GM-CSF, IL-13, TNFα, TGFβ. In addition, cytokines which are known to influence eosinophil survival include IL-3, IL-5, GM-CSF, IL-13, TNFα. Thus, the presence of eosinophil influencing factors which can modulate eosinophil

		Chemo-attractant	activator	survival factor
0-10KDa	**IL-8**	low	x	x?
	Eotaxin	x	x	
	MCP-2	x	x	
	MCP-3	x	x	
	MCP-4	x	x	
	MIP 1α	x	x	
10-30KDa	**IL-1**		x	
	IL-2	x		
	IL-3	low	x	x
	IL-4		x	
	IL-13		x	x
	IL-16		x	
	TGFβ		x	
	TNFα		x	(x)
	GM-CSF	low	x	x
	IFN-γ		x	
	PDGF		x	
30-50KDa	**IL-5**	low	x	x
	IL-12		x	
	VEGF		x	

Table 6-1-7-A; Summary of roles of various cytokines and chemokines which may act as eosinophil chemoattractants, activators and survival factors, classified by molecular mass (modified from Giembycz and Lindsay, 1999). These cytokines and chemokines may be released from HRS cells resulting in eosinophil infiltration, accumulation, activation and survival contributing to the tumour progression in Hodgkin's disease.

Table 6-1-7-B; Summary of reported expression of gene, mRNA and protein secretion of various cytokines and chemokines from clinical biopsy materials and HRS cell lines. (figure legend continue to the next page)

	clinical	L428	L540	KMH2	HDLM2	L591	HDMYZ
IL-1α	R/P(1, 17)	R (14)		R/M (14,9)	R/M (14,9)		R (24)
IL-1β	P (1, 8)						R (24)
IL-2	P (8, 2)						
IL-3	P (8)			M (9)	M (9)		
IL-4	P (4)						
IL-5	R (23)	R (13)		R/M (13)	M (13,9)		R (24)
IL-6	R/P (7,8,11)	R/P (7,11)	P (11)	R/P/M (7,9)	P/M (11,9)		R (24)
IL-7							R (24)
IL-8	R (5,6)	P* (10)	P* (10)	P* (10) /M (9)	P* (10) /M (9)		R (24)
IL-9							R (24)
IL-10	P (2)						
IL-11							
IL-12							
IL-13		R/P (13)		R (13)	R/P (3, 13)		R (13)
IL-14							
IL-15				R (13)			R (13)
IL-16							
GM-CSF	P (8)	R (13)		R/M (13,9)	M (9,13)		
G-CSF	P (8)						
M-CSF				M (9)	M (9)		
TNFα	R/P (1,8)	R/P (7,9)	R (7)	R/P/M (9)	P/M (9)	R (7)	
LT	R (7)	R/P (7)	R/P (7)	R/P/M (7,9)	P/M (9)	R (7)	
TRAC	R (19)	R (19)	R (19)			R (19)	
eotaxin	R/P (18)						
MIG	R/P (18)						
IP-10	R/P (18)						
IFNγ	R (18, 2)						
RANTES	R (18)						
MIPα	R (18)						
MDC	R/P (18,6)						
TGFβ	R (18)	R/P (12)		R/M/P (12,9)	R/M/P (12,9)		
LIF				M (9)	M (9)		
S-ECP	P (22)						

(continued)

Table 6-1-7-B; Summary of reported expression of gene, mRNA and protein secretion of various cytokines and chemokines from clinical biopsy materials and HRS cell lines. Various cytokines and chemokines are measured by the following methods, P; protein measurement by ELISA or immunoblotting, R; mRNA expression by PCR, M; gene expression measured by microarray. * indicates CD40L induced expression. Numbers indicate the reference numbers as follows; 1. Benharrock *et al.* (1996), 2. Dukers *et al.* (2000), 3. Fiumara *et al.*(2001), 4. Ford *et al.* (1984), 5. Foss *et al.* (1996), 6. Hedvat *et al.* (2001), 7. Foss *et al.* (1993), 8. Gause *et al.* (1992), 9. Gruss *et al.* (1992), 10.Gruss *et al.* (1994), 11. Hsu *et al.* (1992), 12. Hsu *et al.* (1993), 13. Kapp *et al.* (1999), 14. McKenzie *et al.* (1992), 15. Mukopadhyaya *et al.* (1987), 16. Newcom and Gu (1995), 17. Perfetti *et al.* (1992), 18. Teruya-Feldstein *et al.* (1999), 19. van den Berg *et al.* (1999), 20. Schwaller *et al.* (1995), 21. Jundt *et al.* (1999), 22. Molin *et al.* (2001), 23. Samoszuk and Nansen (1990), 24. Bargou et al., (1993)

behaviour may have important consequences for the function of eosinophils during HD. In the following paragraphs, those eosinophil influencing factors in HD are discussed.

6.1.8. Chemokines

Chemokines are 7 to 10KDa of molecular mass and contain four distinct cysteine residues that provide the classical subfamily of chemokines. Most of the known chemokines can induce eosinophil migration, activation and recruitment.

Eotaxin

Eotaxin is a strong chemoattractant and promotes tissue eosinophilia in the early stages of inflammation. Interestingly, in HD, eotaxin has been reported controversially from two groups. Jundt *et al.* (1999) examined several biopsy samples and concluded that eotaxin is secreted by fibroblasts in surrounding tissue, but not from HRS cells. On the other hand, Teruya-Feldstein *et al.* (1999 and 2000) have reported HRS cells are indeed capable of producing eotaxin. Another potential source of eotaxin are eosinophils themselves, which they have been shown to be capable of secreting (Nakajima *et al.*, 1998). Despite these contradictory results, it is highly likely *in vivo* that eotaxin may be released from, surrounding tissue, HRS cells or eosinophils to cause the infiltration of eosinophils.

IL-8

As it has been shown in chapter 4, eosinophils produce IL-8 which is controlled by NF-κB pathway. Some HRS cells are also capable of producing IL-8. For example,

in tissue specimen from NS, IL-8 positive cells are commonly found (Gruss *et al.*, 1992). Interestingly in some cases neutrophil infiltration has been observed and correlated weakly between IL-8 production and neutrophil numbers in the tissue (Foss *et al.*, 1996). Unfortunately in the paper by Foss *et al.* (1996) there has been no investigation of whether IL-8 is responsible for eosinophil migration. In the cell lines, HDLM2 and KMH2 express IL-8 gene expression has been reported (Gruss *et al.*, 1992) and CD40L induced IL-8 production has been observed (Gruss *et al.*, 1994).

IL-8 is a pro-inflammatory cytokines and neutrophilic chemoattractant. *In vitro*, IL-8 may have the limited effect as an eosinophil chemoattractant under primed condition (Schweizwe 1994, Sehmi 1993). Eosinophils primed with IL-5 migrate more recruiting towards IL-8 than non primed eosinophils (Schweizwe 1994). But eosinophils from subjects with an eosinophilia migrate to IL-8 without priming with IL-5, but not those from normal subjects (Sehmi 1993). Therefore, IL-8 may not be the direct chemoattractant for eosinophil migration, however, under certain condtions, it enhances the effect of other chemoattractants.

On the other hand, *in vivo*, IL-8 may exert its effect to activate other types of cells which may secret chemoattractant or activator factors to enhance eosinophil recruitment. Therefore, it is possible that IL-8 may not be the direct candidate factor in the recruitment of eosinophils in HD *in vitro*, but may be so *in vivo*. There is no direct evidence suggesting that eosinophils from HRS tumour sites produce IL-8, but if they do, positive feedback production of IL-8 would occur. In other words,

the more eosinophils present at HRS cells, the more IL-8 secretion may occur resulting in more infiltration of eosinophils.

TRAC

The CCR4 receptor binding CC chemokine, TRAC (Thymus and Activation Regulated Chemokine) is known to be a strong activator of T cells. In L428 HRS cell lines and some biopsy material that spontaneous expression of TRAC has been shown (van den Berg *et al.*, 1999). This in turn may recruit Th2 cells and consequently possibly cause the recruitment of eosinophils.

6.1.9. TNFα

The pro-inflammatory cytokine TNFα is commonly expressed in HRS cell lines and can be detected at the mRNA (Foss *et al.*, 1996) and protein level (Xerri *et al.*, 1992, Kretshmer *et al.*, 1990, Hsu *et al.*, 1989). Also in clinical samples, Foss *et al.* (1996) have reported that upon examination of 26 different tissue specimens all were detected as being TNFα positive. TNFα is a powerful eosinophil activator via the NF-κB pathway as it has been shown in chapter 3, 4 and 5. Secretion of TNFα by HRS cells may activate and influence of eosinophils and other cells at tumour sites. In this context TNFα at HRS cells may mimic the situation observed at the inflammatory sites.

6.1.10. Lymphotoxin (TNFβ)

Lymphotoxin (LT) or TNFβ is another cytokine produced by HRS cells. The exact function of LT in HRS cells is still under investigation, however, it may be involved in collagen synthesis in fibroblasts (Semenzato *et al.*, 1990) resulting in

morphogensis of HRS cells. Also LT is a B-cell growth factor (Kehrl *et al.*, 1987) and so may be influential in the development of B-cell origin HRS cells and may be important as a T-cell activator. The direct effect of LT on eosinophils has not been reported. Therefore, the effect of LT on the survival of eosinophils is examined (see results chapter).

6.1.11. IL-5, GM-CSF and IL-3

Both IL-3, IL-5 and GM-CSF are important eosinophil activators. IL-5 and GM-CSF were detected by ELISA in supernatants from L428 and KMH2 but not from HDLM2 (Kapp *et al.*, 1999). On the other hand, controversially, gene expression of IL-5 and GM-CSF has been detected by Northern blotting in HDLM2 and KMH2 by Gruss *et al.* (1992). There have also been reports showing that serum from some patients with HD has the significantly higher levels of circulation of IL-3, GM-CSF and other cytokines (Gause *et al.*, 1992). Further studies are required to ascertain the secretion of these cytokines from HRS cells.

IL-3, IL-5 and GM-CSF have been shown to be weak chemoattractants for eosinophils *in vitro* (Yamaguchi 1988, Hori 1993). *In Vivo*, although there has been shown the correlation between existence IL-5 level and eosinophlia in BAL from asthmatics, it is likely that those cytokines are responsible for eosinophil priming and activation and activation of other inflammatory and surrounding cells.

6.1.12. IL-13

IL-13 is a Th2 cytokine that binds to the alpha chain of the IL-4 receptor. IL-13 has also been reported to be an important chemoattractant for eosinophils (Horie *et al.*,

1997). Fiumara *et al.* (2001) showed RANK (Receptor Activator of NF-κB) ligand (RANKL) upregulates mRNA expression of IL-13, IL-13 receptors, IL-15 and IL-9 in HDLM2, HDMYZ, L428, and KMH2. It has been reported recently that IL-13 and IL-13 receptors prolong HRS cell survival (Kapp *et al.*, 1999, Skinnider *et al.*, 2001), which may contribute to pathogenesis of HD.

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Although there have been numerous investigations, the list (table 6-1-7-B) is still far from complete. Also, methods may be various and to see the completed list of the study on major cytokines and chemokines expression from HRS cells will await future studies.

6.1.13. Eosinophils and other cancers

The involvement of eosinophils in other cancers has been observed in both haematological diseases, such as non-HD leukemia, and in non-haemotological tissues, such as colon, lung and cervix. It is not yet established whether tissue eosinophilia is a contributing factor to the tumour development or beneficial to the host (Reviewed by Samoszuk, 1997). For example, Bethwait *et al.* (1993) showed that patients with eosinophilia in carcinoma of the cervix in stage 1B significantly survived on average 5 years longer compared with the patients with less eosinophil influx in the carcinoma. Also Dalal *et al.* (1992) have reported a better radiation response in eosinophilia patients in cervical carcinoma. Thus, in cervix carcinoma eosinophilia may be a beneficial response.

In most endometrial cancer, degranulating eosinophils have been reported (Blumenthal *et al.*, 2000). This suggests that some eosinophil activation occurs in endometrial cancer but whether this action is beneficial or deleterious to the patient is currently unknown.

To investigate the effect of eosinophilia caused by IL-5, i*n vivo*, an adenocarcinoma cell line, TSA was transfected with IL5 and IL4 genes and injected in syngenic mice (Di Carlo *et al.*, 1998). TSA-IL5 tumour grew progressively in the presence of a large number of eosinophils, suggesting that eosinophilia is contributing to the progress of the disease. On the other hand, TSA-IL4 tumour was rejected in the presence of activated neutrophils, basophils, mast cells, and macrophages. Interestingly, some HRS cell lines inhibit neutrophil migration (Schell-Frederick *et al.*, 1998). If neutrophils have anti-tumour roles, in HD it could be one of the reasons that HRS cells manage to escape the cytotoxic function of the immune system.

In summary it appears that the beneficial effect of eosinophilia in cancer may be dependent on the tumour type and the presence of cytokines and chemokines.

6.1.14. Interaction between HRS cells and eosinophils- phagocytosis by HRS cells?

In vivo, there are numerous interactions between HRS cells and other cell types. Possibly the most common interaction is the adherance of HRS cells with accumulated inflammatory cells. This action may further influence activation status

of the accumulated inflammatory cells. Another possibility of physical interaction is phagocytosis or emperipolesis, in which there is passage of a cell into the cytoplasm of another cell. In fact, in the 1970s and 80s some researchers had indeed reported by microscopic observation that HRS cells are capable of phagocytosis or emperipolesis of granulocytes and other cells. For example, Brooks *et al.* (1979) reported that HRS cells engulfed some neutrophils but not eosinophils. Similarly, Quaglino *et al.* (1977, 1979) demonstrated phagocytosis (or emperipolesis) of erythrocytes, lymphocytes (also Kay *et al.*, 1975) and granulocytes (unknown whether they were neutrophils or eosinophils). Also, Poppema *et al.*, (1978) and Bernuau *et al.*, (1977) showed the existence of Ig and human serum albumin within HD cells, suggesting cell wall 'disruption'. Finally, *in vitro*, phagocytosis of *Candida albicans* by HDLM-1 and KIM-H2 (Hsu *et al.*, 1990), latex and ink particles by SU/RH-HD-1 (Olsson *et al.*, 1984) and HuT11 (Roberts *et al.*, 1987) has been reported. The main purpose of these reports were to identify the origin of the HRS cells by microscopic observation before all the new techniques, to identify the phenotype and surface markers of HRS cells, were available. Therefore, seeing phagocytosis or emperipolesis of HRS cells, in some reports, HRS cells were determined as macrophage origin. However, except in the clinical biopsy samples, in only established cell lines, HDMYZ has been suspected as macrophage origin (Bargour *et al.*, 1993).

The aims of this chapter are;

- To investigate whether supernatants derived from HRS cells could influence eosinophil and neutrophil survival.

- To compare the effect of supernatants from HRS cells with IL-5 or GM-CSF on the survival of eosinophils.
- To examine whether eosinophil survival effects are dependent on the NF-κB pathway.
- To investigate the molecular mass of substance x by molecular mass separation techniques.
- Observation of phagocytosis of eosinophils by L540.

- However, in here, to identify the substance x is not our aim and intention.

6.2. - 6.15. Results

6.2. Experimental methods

As it has been discussed in the introduction, the causes of eosinophilia at HRS cells may be due to migration, activation and survival of eosinophils. Although there is more than one cause of eosinophilia, our initial study was to investigate whether HRS supernatants influence eosinophil survival and apoptosis.

To investigate the effect of eosinophil survival by HRS cells, freshly isolated eosinophils were cultured with the supernatants of various HRS cell lines without cells (experiments 6.2. to 6.12.) and co-incubation with HRS cells (experiments 6.13. to 6.15.). After the eosinophils were cultured for the time indicated in each experiment, eosinophils survival was assessed by microscopic observation. The aim of culturing eosinophils with supernatants of HRS cells is to understand the nature of 'substance x' on eosinophil survival. In contrast, co-incubation with eosinophils and HRS cells is to investigate the interaction between the two different types of cells *in vitro*.

In the following sections, supernatants were harvested from various HRS cell lines, L428, L540, L591, KMH2, HDLM2 and HDMYZ (see their origin and nature in table 6-1-4). Each supernatant was incubated at 10% of the final volume with eosinophils (3.0 $x10^6$ cells/ml) and neutrophils (5.0 $x10^6$ cells/ml). After the indicated incubation times, eosinophils and neutrophils were harvested and morphologically assessed for their survival rate. The reasons for assessing the number of the cells surviving instead of undergoing apoptis is due to the long time period of the culture, it was difficult to quantify apoptotic cells since they had

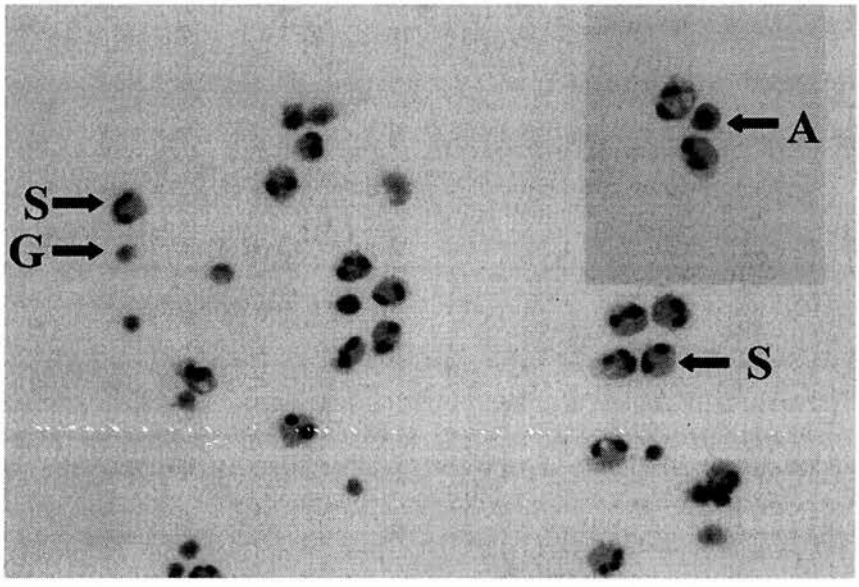


Figure 6-2; Typical view of eosinophils treated with supernatant L540. Eosinophils were cultured with 10% of final volume of supernatant of L540 for 12 days. Survival rate was morphologically assessed by percentage. Survived cells (S), ghost cells (G), apoptotic cells (A) are indicated. Magnification x 63.

turned into 'ghost cells' or cell fragments. Thus, in this chapter, only healthy living cells were morphologically counted in the cell population. For example, figure 6-2 shows a typical light microscopy view of healthy eosinophils treated with L540 supernatant at 12 days. The arrow indicates the dead 'ghost' cells (G), which are difficult to determine whether it is apoptotic (A) or necrosis. Therefore, survival rate was assessed instead of apoptosis. In addition, throughout the experiments, the number of the cells/ml was counted to confirm the number of the cells in the culture. In the following experiments, control condition indicates that it contains 10% of 'blank' medium, in which all the HRS cell lines (except HDMYZ) are kept. Only HDMYZ was kept at 20% of FCS in RPMI1640 instead of 10%.

Instead of using 10% of supernatant medium from HRS cell lines to treat neutrophils, 5% or 20% of the supernatants were also tested (figure 6-4-b). There was no great difference between 10% and 20% of supernatants on both eosinophils and neutrophil survival. On the other hand, 5% of the supernatants had about half effect on granulocyte survival that of 10%. In summary 10% was determined to be the optimal percentage for this study.

In addition, supernatants were also taken from the different passages of HRS cell lines on separate occasions and eosinophil survival was examined. Supernatants obtained from different passages did not appear to make any difference (data not shown).

6.3. The effect of supernatants obtained from HRS cell lines on eosinophil survival.

To examine the different effects of supernatants from various HRS cell lines on eosinophil survival, eosinophils were cultured with 10% of total volume of supernatants from various HRS cell lines. After 7 days, cells were harvested and the survival of cells were morphologically assessed.

The results are shown in figure 6-3 and 6-3-B. In here, control is indicated as 'C' and contains 10% of 'blank' medium (10% FCS in RPMI1640) in which all the HRS cell lines, except HDMYZ, are kept. The eosinophils treated with L428 and L540 showed significantly prolonged longevity, compared to other conditions and GM-CSF. As it has been demonstrated previously the rate of constitutive eosinophil apoptosis *in vitro* is normally about between 4 to 7 days. Thus, it is striking that eosinophil survival was induced by the supernatants of L540 (about 7 fold compared to C) (see figure 6-3-B) and L428 (about 5 fold compared to C) for 7 days. Furthermore, in some occasions, some eosinophils survived up to 28 days when treated with L540 supernatant (data not shown).

On the other hand, supernatants from other cells lines, L591, KMH2, HDLM2 and NDMYZ did not show any significant survival effect compared to control conditions. The difference between the most and least effective supernatants is not known. To examine the origin and nature of the cells (see table 6-1-4) and generation of cytokines and chemokines (see table 6-1-7-B), there appears be no correlation. These experiments were repeated at least three times from different

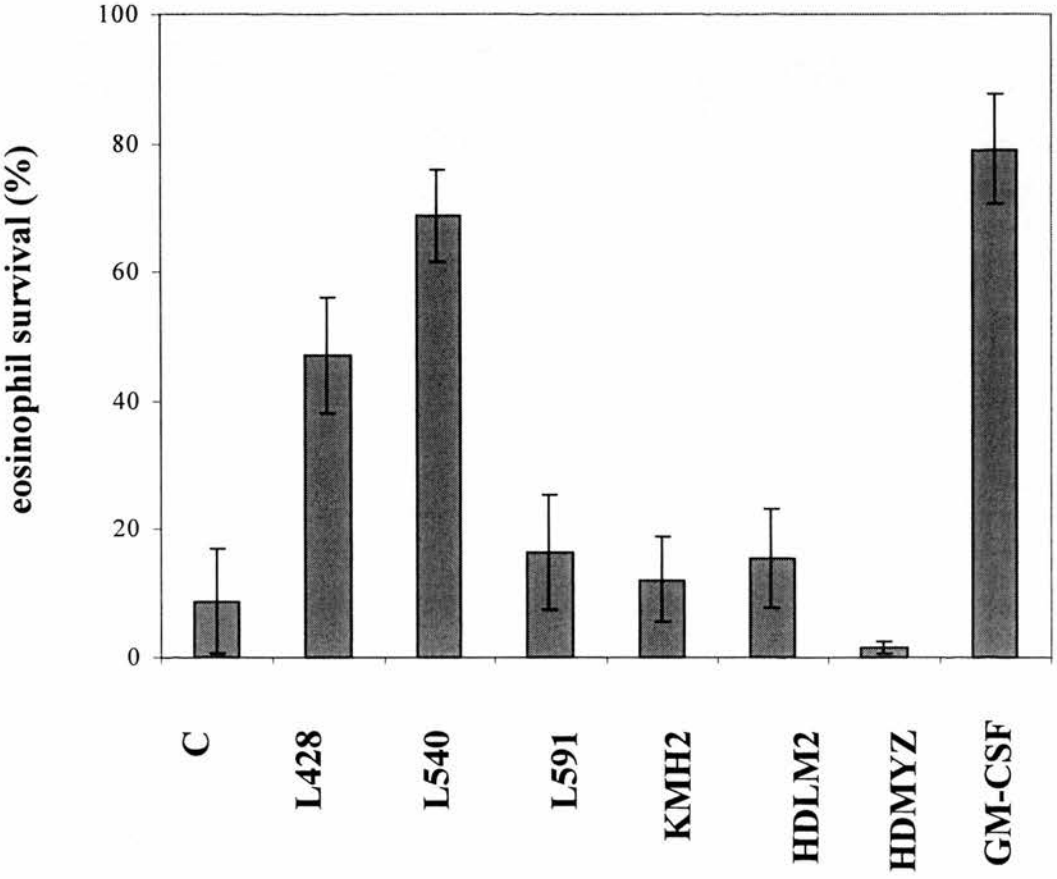
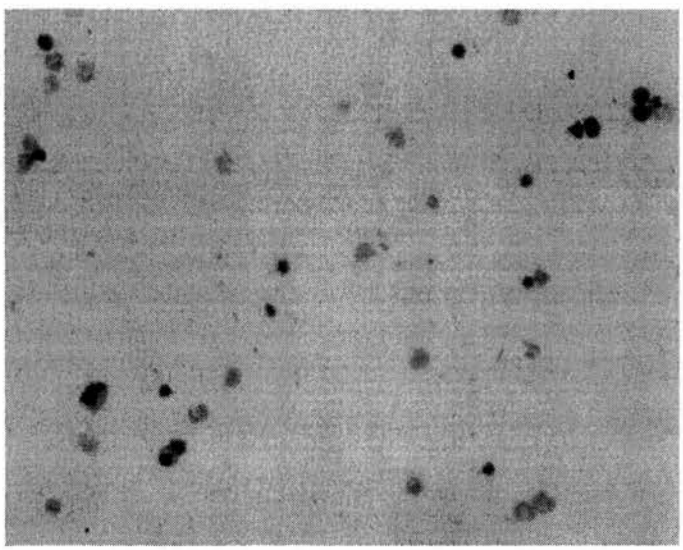


Figure 6-3; Effect of supernatants from HRS cell lines on eosinophil survival. Purified eosinophils (3.0 x 10^6 cells/ml), cultured in Iscove's DMEM containing 10% autologous serum, were treated with 10% of supernatants from different types of HRS cell lines and GM-CSF (500ng/ml)and kept at 37°C for 7 days. 'C' indicates the control condition with 'blank' medium, containing 10% FCS in RPMI1640, in which all the HRS cell lines were kept, except HDMYZ. After 7 days cell survival was morphologically assessed. All the values represent mean with standard error from four different donors. Supernatant from L540 and L428 showed significant differences from control indicated as * where $P<0.01$.

Control; no stimuli



L540 supernatant

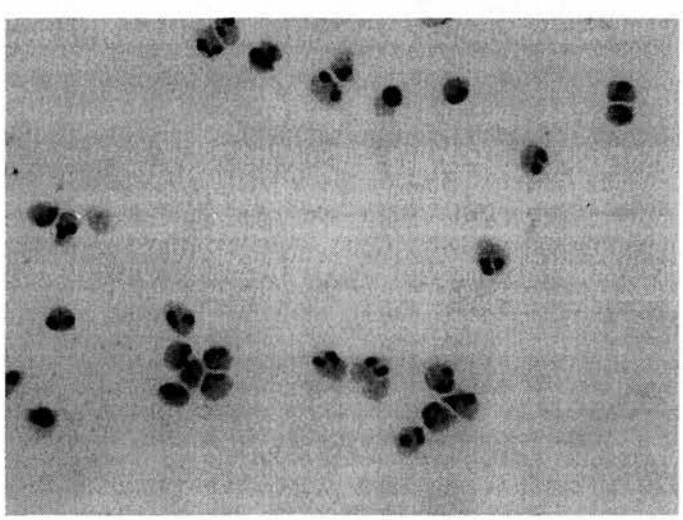


Figure 6-3-B; L540 supernatant treated eosinophils. Eosinophils were cultured with supernatant from L540 cell line for 7 days (panel below). Panel above shows the control condition, without any treatment for 7 days. (Magnification x 63).

generations/passage of the HRS cell culture. They all show a similar trend in that L428 and L540 have a greater effect on eosinophil survival.

Since L540 and L428 showed the best eosinophil survival effects, both supernatants were selected for further experiments.

Dose response curve shows (figure 6-4- B) shows the correlation between eosinophil survival and dose of supernatant from different HD cell lines. However, 10 % of supernatant was chosen as the concentration for the following experiments.

Summary

- L428 and L540 supernatants showed significantly prolonged survival of eosinophils assessed after 7 days culture.
- L591, KMH2, HDLM2, HDMYZ supernatants did not show any significant effect on eosinophil survival after 7 days culture.
- The reasons for the difference between the cell lines which had a 'survival effect' (L428 and L540), and those that had 'no-survival effect' (L591, KMH2, HDLM2, HDMYZ) are not known.
- Dose response curve shows (figure 6-4- B) shows the correlation between neutrophil survival and dose of supernatant from different HD cell lines.

6.4. The effect of supernatants obtained from HRS cell lines on neutrophil survival.

In HD, neutrophils are also occasionally observed at the tumour, although the number of neutrophils are less notable than eosinophils. Similarly to eosinophils, the causes of neutrophil accumulation at the tumour is unknown.

To investigate whether supernatants of HRS cell lines may also influence neutrophil survival, the same type of experiments as outlined as above (6.3.) were performed on neutrophils incubated for 24 hours culture, at the time approximately 60 - 90% of the non-treated cells spontaneously become apoptotic. All the experimental methods were the same as above.

The results are shown in figure 6-4 and 6-4-B. Similar to eosinophils, L428 and L540 supernatants exerted significant neutrophil survival. Compared to the control condition, L428 supernatant induced about 6 fold and L540 was about 7 fold greater percentage in survival. Remarkably, neutrophils treated with L540 supernatant occasionally survived up to 7 days (data not shown). This is a phenomenal result, to be able to keep neutrophils alive for 7 days *in vitro*.

Although, the effects are not as great as L428 and L540, L591 and KMH2 supernatants showed efficient neutrophils survival effects compared to the control condition. Both HDLM2 and MDMYZ supernatants had very little effect on neutrophil survival compared to control condition.

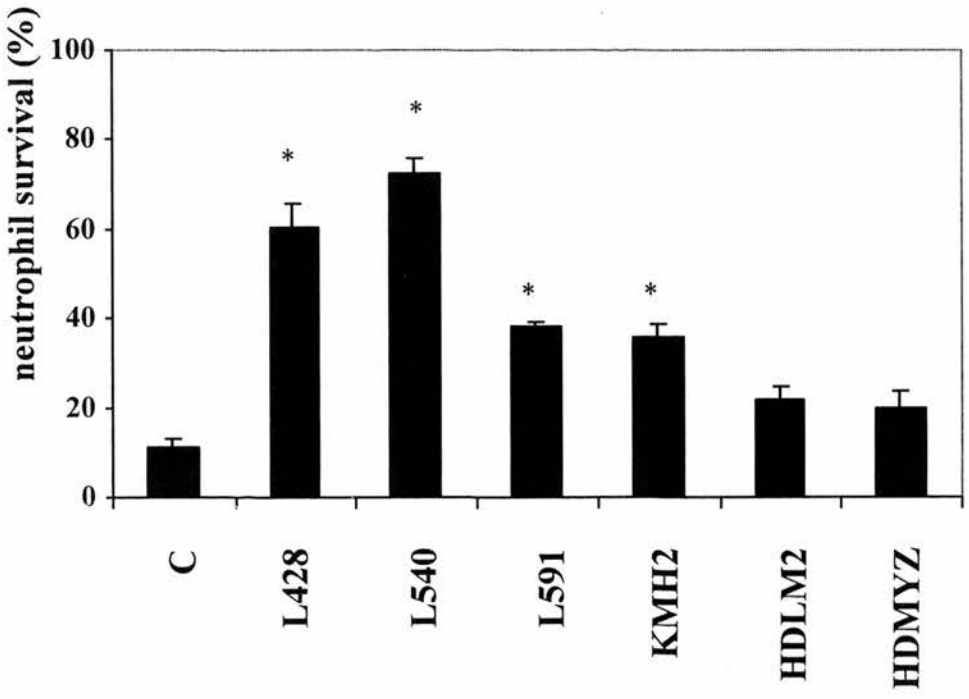


Figure 6-4; Effect of supernatants of HRS cell lines on neutrophil survival. Purified neutrophils (5.0 x 10^6 cells/ml), cultured in Iscove's DMEM containing 10% autologous serum, were treated with 10% of supernatants from different types of HRS cell lines and kept at 37°C for 24 hours. 'C' indicates the control condition with 'blank' medium, containing 10% FCS in RPMI1640, in which all the HRS cell lines were kept, except HDMYZ. After 7 days cell survival was morphologically assessed. All the values represent mean with standard error from four different donors. Supernatant from L540 and L428 showed significant differences from control indicated as * where $P<0.01$.

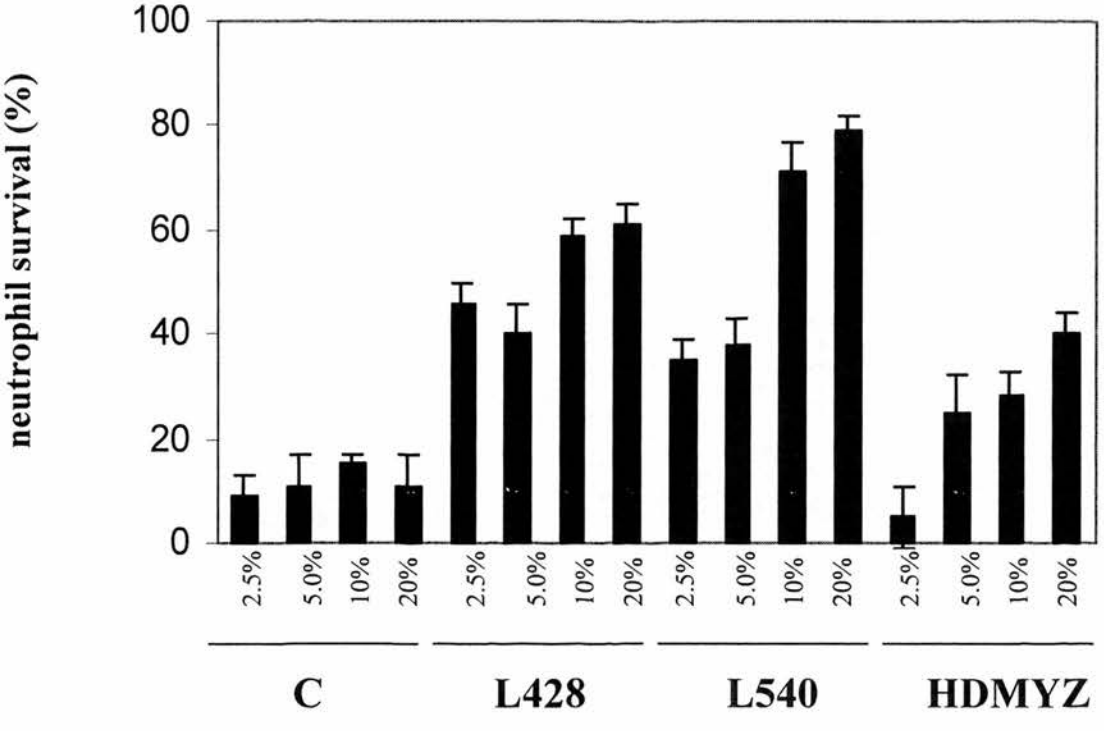


Figure 6-4-B; Effect of dose response on supernatants of HRS cell lines on neutrophil survival. Purified neutrophils (5.0 x 10^6 cells/ml), cultured in Iscove's DMEM containing 10% autologous serum, were treated with 2.5%, 5.0%, 10% and 20% of supernatants from different types of HRS cell lines and kept at 37°C for 24 hours. 'C' indicates the control condition with 'blank' medium, containing 2.5%, 5.0%, 10% and 20% FCS in RPMI1640, in which all the HRS cell lines were kept, except HDMYZ. After 7 days cell survival was morphologically assessed. All the values represent mean with standard error from four different donors.

Although neutrophils and eosinophils are derived from a common myeloid precursor, they do have a different cellular metabolism and are activated differently, therefore, different stimuli would cause different effects on their survival or apoptosis. The supernatants of L591, KMH2, and HDMYZ may be more efficient at inducing survival effect in neutrophils compared to eosinophils. Although the incubation times were different in each cell type, therefore, it may not be a straight comparative study, however, if each supernatants are compared to the control condition, the different effect is clearly significant. In those supernatants, they may contain a substance(s), which is specific to neutrophil survival but not eosinophils. In contrast, since L428 and L540 exert their effects on neutrophils survival, they may also contain substances that induce survival in both neutrophils and eosinophils.

Dose response curve shows (figure 6-4- B) shows the correlation between neutrophil survival and dose of supernatant from different HD cell lines.

Summary

- L428 and L540 supernatants exerted prolonged neutrophil survival.
- L428 and L540 may contain common survival factors for both neutrophils and eosinophils.
- L591, KMH2, HDLM2 and HDMYZ supernatants induce neutrophil survival but could not cause eosinophil survival.
- L591, KMH2, HDLM2 and HDMYZ may produce neutrophil specific survival factors.

- Dose response curve shows (figure 6-4- B) shows the correlation between neutrophil survival and dose of supernatant from different HD cell lines.

6.5. Supernatant of L540 is more efficient at prolonging eosinophil survival than IL-5 and GM-CSF.

To assess the effect of L540 supernatant on eosinophil survival compared to other known eosinophil survival factors, IL-5 and GM-CSF were chosen for comparison. Both IL-5 and GM-CSF share the β subunit of receptors and trigger tyrosine kinases pathways to influence survival (Yousefi *et al.*, 1996, Paxdrak *et al.*, 1997) and delay apoptosis (Tai *et al.*, 1991, Stern *et al.*, 1992, Yamaguchi *et al.,* 1991)

Eosinophils were cultured with IL-5 (50ng/ml) and GM-CSF (500ng/ml) for 7 days and the rate of survival was assessed morphologically. The results are shown in figure 6-5. For 7 days culture, L540 showed on average a slightly better effect on eosinophil survival than IL-5 and GM-CSF alone.

This result may suggest that supernatant from L540 contains a 'stronger' survival factor than a single incubation of 50ng/ml IL-5 or 500ng/ml GM-CSF. Previously, we had performed concentration-response experiments of IL-5 and GM-CSF on eosinophil survival and concluded that these concentration used were optimal. Regardless of the existence of IL-5 and GM-CSF in the supernatant of L540, there may be the some other factors that induce eosinophil survival.

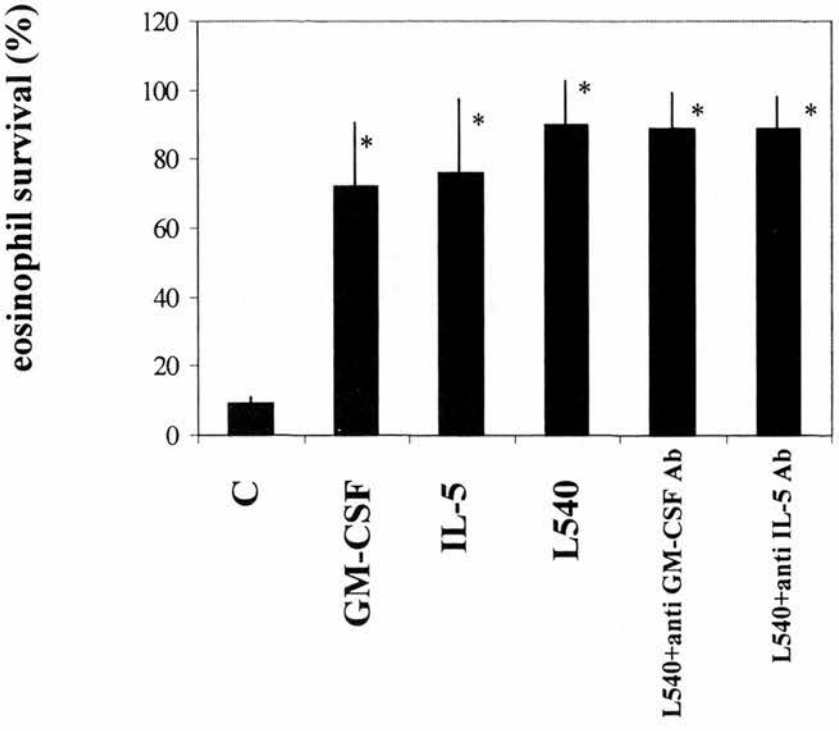


Figure 6-5; Effect of GM-CSF, IL-5, anti-GM-CSF antibody, anti-IL-5 antibody and supernatant from L540 HRS cell line on eosinophil survival. Purified eosinophils (3.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum were treated with GM-CSF (500ng/ml), IL-5 (50ng/ml) and 10% of supernatant from L540 HRS cell line and kept at 37°C for 7 days. 'C' indicates the control condition with 'blank' medium, containing 10% FCS in RPMI1640, in which all the HRS cell lines were kept, except HDMYZ. After 7 days cell survival was morphologically assessed. All the values represent mean with standard error from four different donors. Supernatant from L540 showed the significant differences from control indicated as * where $P<0.01$.

Preliminary, using anti-IL-5 and GM-CSF antibodies, neutralising experiment were performed. However, there was no effect on supernatant of L540 to inhibit its effect (data not shown). Unfortunately, this experiment could not be continued. (See discussion 6.16.1. for more details.)

Summary

- Supernatant of L540 exerted a slightly better effect on eosinophil survival assessed 7 days culture compared to IL-5 and GM-CSF.

6.6. Lymphotoxin on eosinophil survival

Lymphotoxin (LT) or TNFβ is produced in abundance by almost all the HRS cell lines (Foss *et al.*, 1993, Gruss *et al.*, 1992). In particular in the supernatant derived from L540 LT is present. LT is involved in the activation of T cells and B cells and possibly HRS cells themselves. Little is known of the effect of LT on eosinophil function and there have been no prior reports on eosinophils survival or apoptosis by LT. To investigate if LT is playing any roles on eosinophil survival, LT was cultured with eosinophils and survival was assessed.

The results are shown in figure 6-6. For 7 days culture, at the different concentrations of LT used no significant effect on eosinophil survival was observed. Although HRS cells secrete substantial amounts of LT, it appears that there is no effect of LT on eosinophils survival. In the supernatant of L540, other substances may be the direct cause of eosinophil survival. LT may have other roles to orchestra

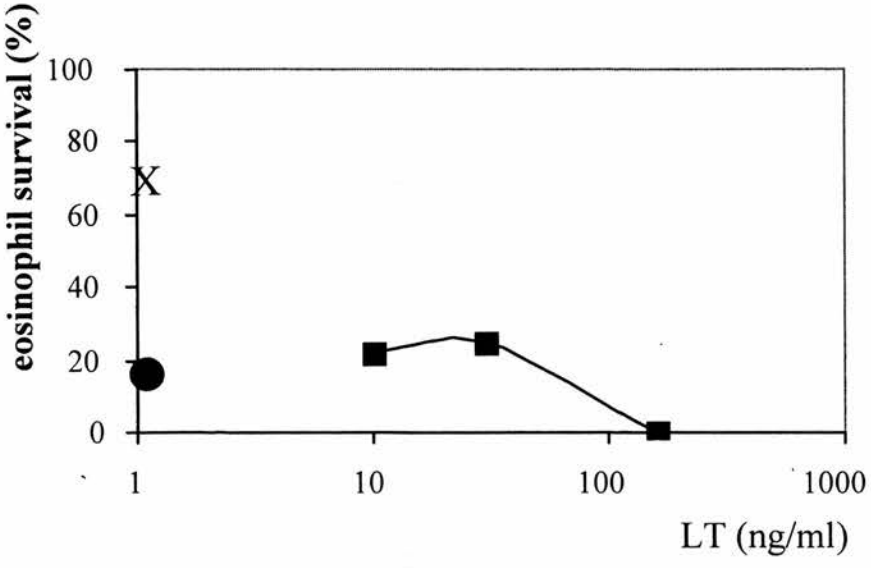


Figure 6-6; Effect of Lymphotoxin (LT) on eosinophil survival.
Purified eosinophils (3.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum were treated with LT for 7 days. On Y-axis, ● indicates as a control condition without any treatment. X indicates as a 10% L540 supernatant-treated eosinophil survival. After 7 days cell survival was morphologically assessed. This is a representative experiment.

some other inflammatory cells at tumour but not the direct cause of eosinophil survival.

Summary

- LT does not appear to have any effect on eosinophil survival.

6.7. LT on neutrophil survival

LT was previously shown to enhance neutrophil phagocytosis (Shalaby *et al.*, 1985) However, there are no reports on the effect of LT on neutrophil apoptosis or survival. To investigate the effect of LT on neutrophil survival, LT was cultured with neutrophils for 24 hours and 48 hours and survival assessed.

The results are as shown in figure 6-7. After 24 hours culture, there was a slight effect of LT on neutrophil survival especially at the higher concentration. At 48 hours, although the survival rate was substantially lower, some surviving cells were observed, while there was none in the control condition. Therefore, LT may also have a slight effect on neuturophil survival at 48 hours. However, at both time points, the survival rate was not as high as the supernatant of HRS cells (e.g., L540). LT is unlikely to be the direct cause of neutrophil survival. Thus, in the supernatant of L540, other factors may be responsible for neutrophil survival.

Summary

- LT may have a slight effect on neutrophil survival.
- LT is unlikely to be the direct cause of neutrophil survival in the supernatant of HRS cells.

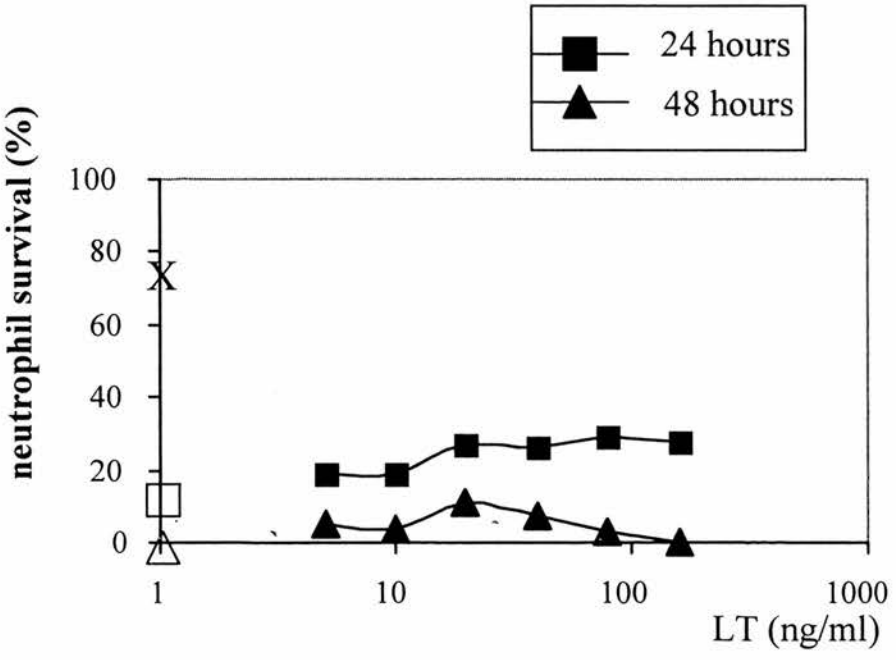


Figure 6-7; Effect of Lymphotoxin (LT) on neutrophils survival.
Purified neutrophils (5.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum were treated with LT for 24 and 48 hours. On Y-axis, □ indicates the control condition without any treatment at 24 hours. X indicates as a 10% L540 supernatant-treated eosinophil survival. At 48 hours under control conditions without any treatment (Δ), no cells survived. After 24 and 48 hours cell survival was morphologically assessed. This is a representative experiment.

6.8. Abrogation of L540-supernatant-induced eosinophil survival by NF-κB inhibitors.

As it has been shown in the previous chapters, the NF-κB pathway plays an essential role in eosinophil survival. To investigate the involvement of NF-κB in L540-supernatant-induced eosinophil survival, eosinophils were cultured with NF-κB inhibitors in the presence of supernatants of L540. The NF-κB inhibitors used were Mg132 (20μM) and TAT-IκBα32,36 (30μg/ml) and as a control GST-TAT (30μg/ml).

The results from two different donors (panel A and B) are shown in figure 6-8. Firstly, freshly isolated eosinophils were treated with 10% of L540 supernatant for 5 days. Then, NF-κB inhibitors were added to the culture. In panel A in figure 6-8, there is a clear inhibition of eosinophil survival by TAT-IκBα32,36. As shown in chapter 5, the efficiency of TAT-IκBα32,36 may be donor-dependent. However, in these experiments, using three different donors, all showed inhibition of L540-supernatant-induced eosinophil survival. Similarly, graph B in figure 6-8 shows that L540-supernatant-induced eosinophil survival was inhibited by TAT-IκBα32,36 and Mg132 in 7 days of culture. Mg132 induced a dramatic killing effect on eosinophils. GST-TAT showed a small inhibition of the survival effect induced by L540 supernatant.

By using different NF-κB inhibitors, these results show that L540-supernatant-induced eosinophil survival is abrogated by inhibiting NF-κB. Thus, the NF-κB

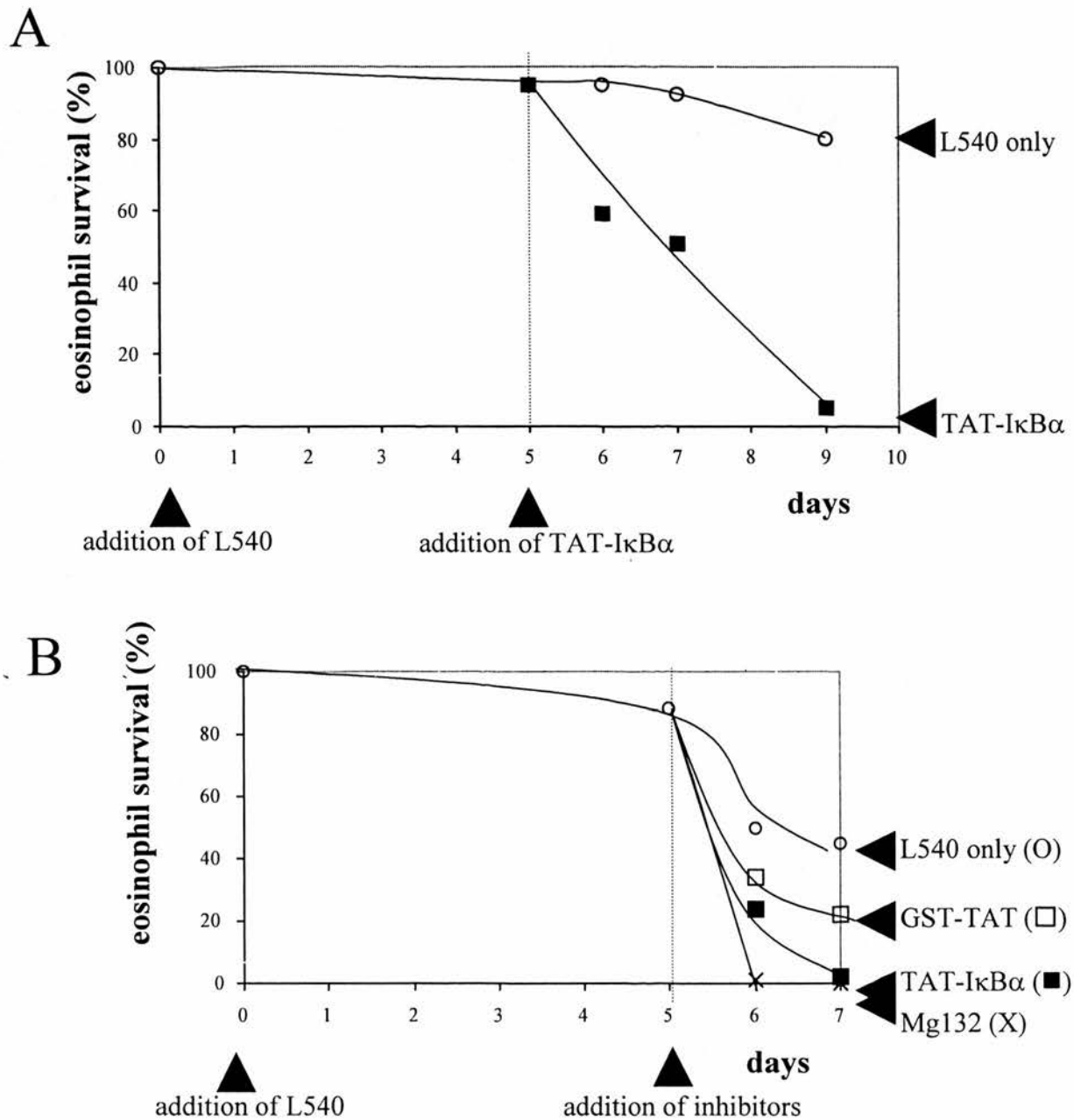


Figure 6-8; The effect of incubation with various NF-κB inhibitors on eosinophils which had been previously treated with the supernatant of L540 HRS cell line. Purified eosinophils (3.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with 10% of supernatants from L540 HRS cell lines for 5 days. Then various NF-κB inhibitors were added. Eosinophils used in panel A and B are from different donors. From day 5 cells survival was morphologically assessed. These are represent experiments out of three different experiments performed on separate occasions.

pathway appears to play an important role in controlling L540-supernatant-induced eosinophil survival.

Summary

- TAT-IκBα32,36 inhibits L540-induced eosinophil survival.
- Mg132 inhibits L540-induced eosinophil survival.
- Inhibition of NF-κB abrogates L540-induced eosinophil survival.

6.9. Abrogation of eosinophil survival by NF-κB inhibitors cannot be reversed by supernatant from L540.

In the next series of experiments, NF-κB inhibitors were cultured with eosinophils before the addition of L540 supernatant. The aim of these experiment was to investigate whether the effect of L540 supernatant can 'rescue' eosinophils, which had been previously treated with NF-κB inhibitors. TAT-IκBα32,36 (30μg/ml), GST-TAT (30μg/ml) and Mg132 (20μM) were added to eosinophils culture for 24 hours and then supernatants from L540 was added.

Purified eosinophils were cultured with NF-κB inhibitors or un-treated (indicated as 'C'), for 24 hours, then, supernatant of L540 were added (filled circle) or none (open triangle). In control condition 'C', addition of supernatant of L540 'rescued' eosinophils from constitutive apoptosis. For example at day 4, the survival rate of untreated eosinophils was about 15%, whereas L540 supernatant treated cells showed a good survival rate (60%). This is significantly different from cells without any treatment. Similar effects were seen in eosinophils that were treated with GST-

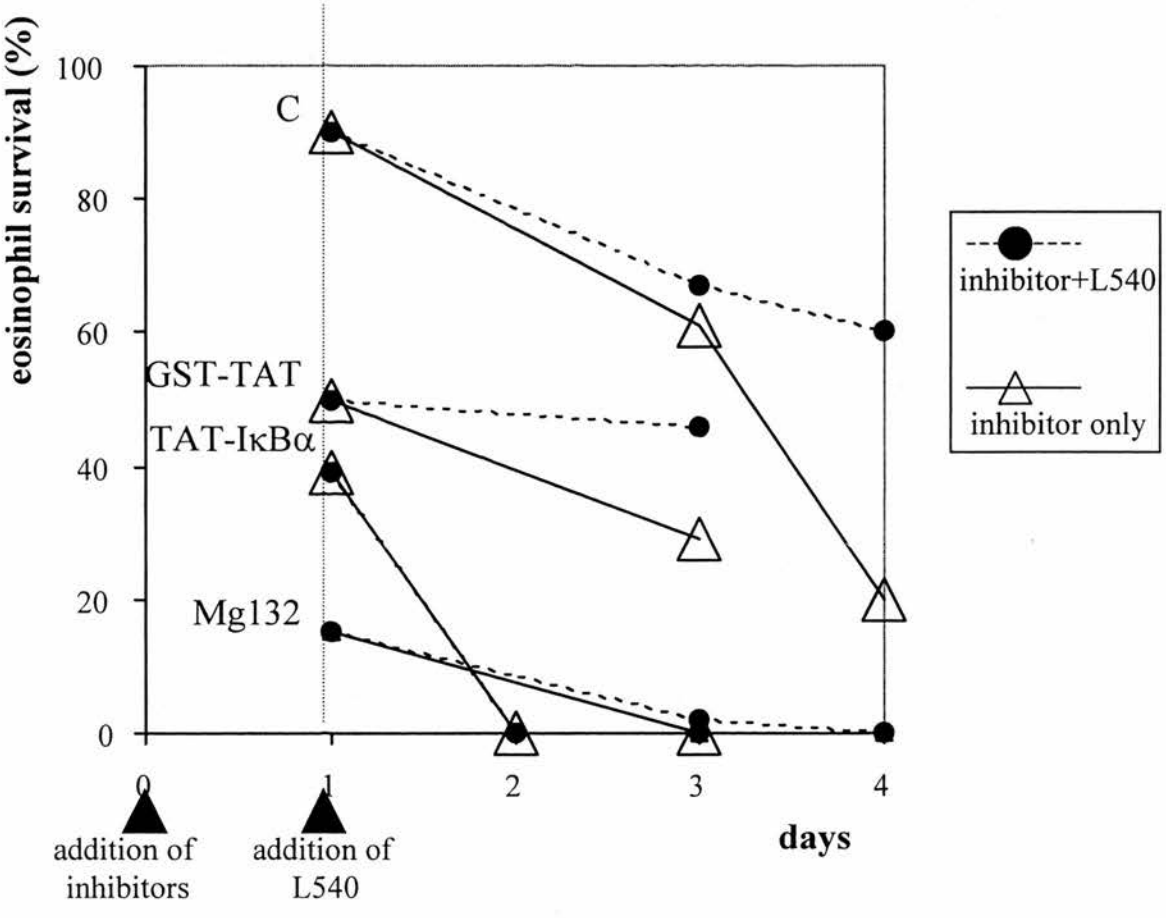


Figure 6-9; The effect of incubation with various NF-κB inhibitors on eosinophils for a day before treatment with the supernatant from L540 HRS cell line. Purified eosinophils (3.0 x 10^6 cells/ ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with various NF-κB inhibitors for one day. Then 10% of supernatants from L540 HRS cell line were added to those cultures. 'C' indicates the control condition without any inhibitors. Eosinophil survival was morphologically assessed from day one. These are represent experiments out of three different experiments performed on separate time.

TAT. GST-TAT+L540 treated cells also showed a better survival rate compared to the GST-TAT only. These results indicate that L540 supernatant can prolong eosinophils survival after one-day of eosinophil culture.

In contrast, incubation with NF-κB inhibitors for 24 hours, eosinophils underwent apoptosis. For example, the survival rate treated with Mg132 was about 15%, TAT-IκBα was about 40% in 24 hours culture. However, after the addition of L540 supernatant, in both conditions L540 supernatant could not 'rescue' the inhibition of eosinophil survival. These results suggest that eosinophil survival may be regulated by the NF-κB pathway. Once the NF-κB pathway is inhibited by Mg132 and TAT-IκBα32,36, L540 supernatant cannot reverse the fate of the cells.

Summary

- Supernatant from L540 exerts its effect on survival on one-day-old (untreated) eosinophils.
- Supernatant from L540 exerts its effect on eosinophil survival which had been previously treated with GST-TAT for 24 hours.
- Supernatant from L540 does not exert its effect on survival on eosinophils, which had been previously cultured with TAT-IκBα32,36 and Mg132 for one day.
- These data suggest the involvement of the NF-κB pathway in L540-supernatant-induced eosinophil survival.

6.10. Separation of supernatant of L540 by molecular mass and their survival effect on eosinophils.

To identify substance x will require extensive investigation. (For further discussion of screening process is discussed in the discussion 6.16.1.) However, to attempt to determine the possible candidates for substance x, isolation of the supernatant according to the molecular mass by centrifugation was performed (see methods 2.12.3.). The supernatants were isolated as; x<10KDa, 10KDa<x<30KDa, 30KDa<x<50KDa, 50KDa<x. Each fractions of supernatants were cultured with eosinophils for up to 10 days and survival rate was assessed.

The results are shown in figure 6-10. 'L540' indicates the supernatant without centrifugation. The fraction smaller than 10KDa (x<10KDa) showed very low eosinophil survival in 5 days and none at 10 days. Although the precise content of supernatant less than 10KDa is not known, it is likely to contain chemokines, lipids, peptides etc. Interestingly, chemokines which are known eosinophils activators, chemoattractants and survival factors are usually less than 10KDa (see table 6-1-7-A for summary). Thus, it may be possible to eliminate these low molecular weight produces from the list of candidates for substance x. Substances bigger than 10KDa are the likely eosinophil survival factors.

The fraction between 10KDa and 30KDa (10KDa<x<30KDa) showed less survival effect in both 5 and 10 days compared to the whole L540 supernatant. The fraction between 30KDa and 50KDa (30KDa<x<50KDa) showed similar survival effect to the whole supernatant L540 in both days.

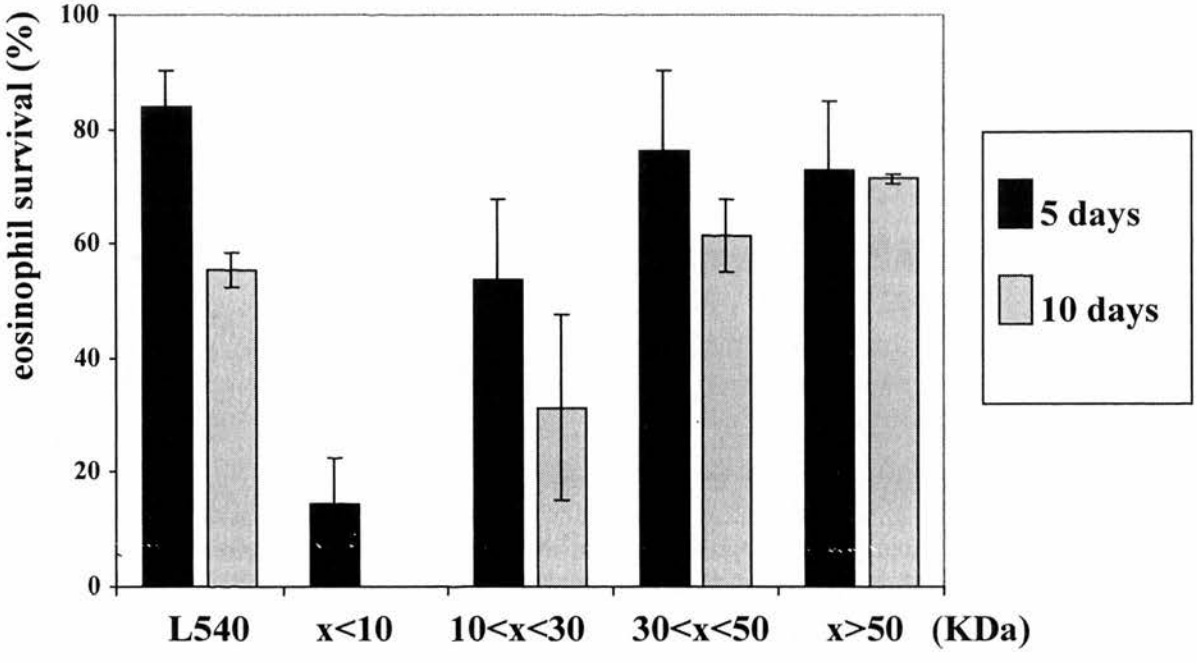


Figure 6-10; The effect of the incubation with the supernatant of L540 HRS cells, in which the contents of the supernatants had been separated according to molecular mass. Purified eosinophils (3.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with 10% of supernatants from L540 HRS cell line in which the contents of the supernatant had been separated according to molecular mass by a series of centrifugations. 'L540' indicates the control condition which has been treated with whole L540 supernatant (10%). Then eosinophil survival after 5 days or 10 days were assessed morphologically. All the values represent mean with standard error of three different donors.

The fraction, which is bigger than 50KDa (x>50KDa) showed good survival effects, similar to uncentrifuged supernatant of L540 after 5 and 10 days. Even, after 10 days culture, x>50KDa showed a better survival effect than the whole supernatant of L540. This indicates the presence of powerful eosinophil survival factors in the x>50KDa fraction.

Summary

- In L540 supernatant, the fraction, which is smaller than 10KDa does not contain the eosinophil survival factor.
- In L540 supernatant, the fraction, which is bigger than 50KDa may contain the strong eosinophil survival factor.

6.11. Separation of supernatant of L428 by molecular mass and their survival effect on eosinophils.

The same experiment as above (6.10.) was performed using the supernatant derived from L428. The results are shown in figure 6-11. Compared to whole L540, whole L428 exerted less eosinophil survival after 10 days culture. With the exception of this time point, a similar trend was seen with L428 culture compared to L540. The fraction less than 10KDa (x<10KDa) has dramatically reduced survival effect assessed after 5 days and none after 10 days. Again, the possibility of chemokines as the eosinophil survival factors can be ruled out. On the other hand, fractions bigger than 30KDa (30KDa<x<50KDa and x>50KDa) showed better eosinophil survival especially after 5 and 10 days. Therefore, the fractions bigger than 30KDa

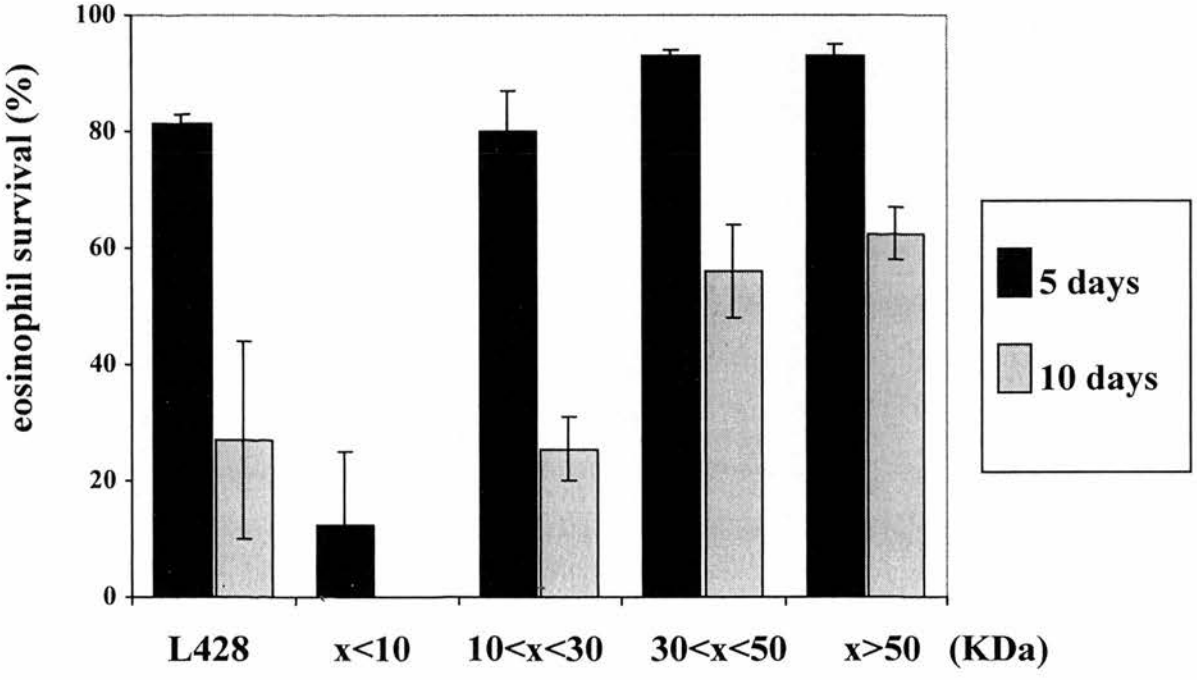


Figure 6-11; The effect of the incubation with the supernatant of L428 HRS cells, in which the contents of the supernatants had been separated according to molecular mass. Purified eosinophils (3.0 x 10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum, were treated with 10% of supernatants from L428 HRS cell line in which the contents of the supernatant had been separated according to molecular mass by a series of centrifugations. 'L428' indicates the control condition which has been treated with whole L428 supernatant (10%). Then eosinophil survival after 5 days or 10 days were assessed morphologically. All the values represent mean with standard error of three different donors.

contains the eosinophils survival factor in the supernatant of L428. The fraction between 10 and 30KDa (10KDa<x<30KDa) showed very similar rates of eosinophil survival compared to the whole L428.

Summary

- In L428 supernatant, the fraction, which is smaller than 10KDa does not contain the eosinophil survival factor.
- In L428 supernatant, the fraction, which is bigger than 30KDa contains the eosinophil survival factor.

6.12. Separation of supernatant of L540 by molecular mass and heat shocked

To investigate further the isolated fraction from supernatant of L540 (6.10.), the fractions were examined to see whether they contained heat resistance substances or not. Each fractions were heat shocked at 40°C, 60°C, 80°C and 100°C for 15 minutes and cultured (10% of final volume) with eosinophils for up to 10 days and the survival rate was assessed. The results are shown in figure 6-12.

In the whole supernatant, in 5 days culture, despite heat treatment for the different temperatures, there was no significant differences on eosinophil survival. In contrast, after 10 days culture, heat treated supernatants failed to prolong survival compared to the non-heat-treated condition. This indicates that some survival factors in the supernatant of L540 are heat unstable.

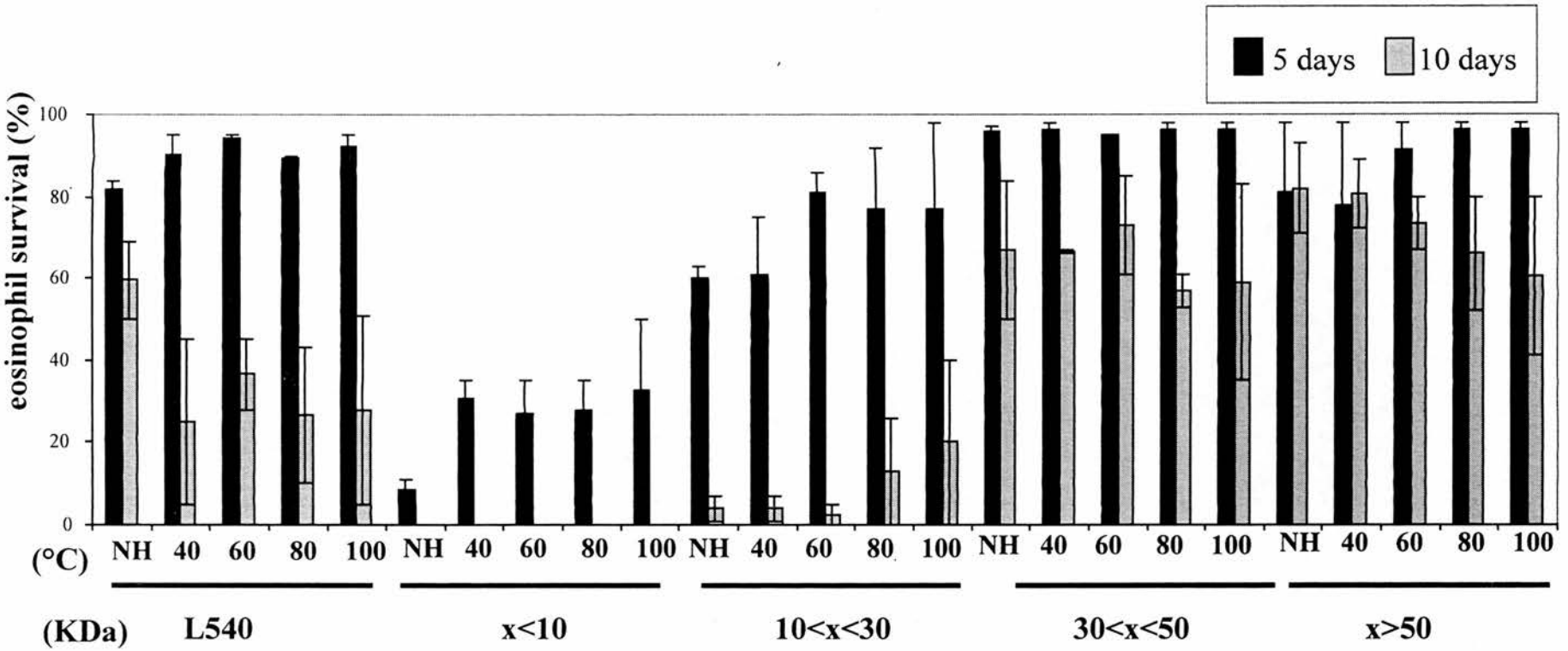
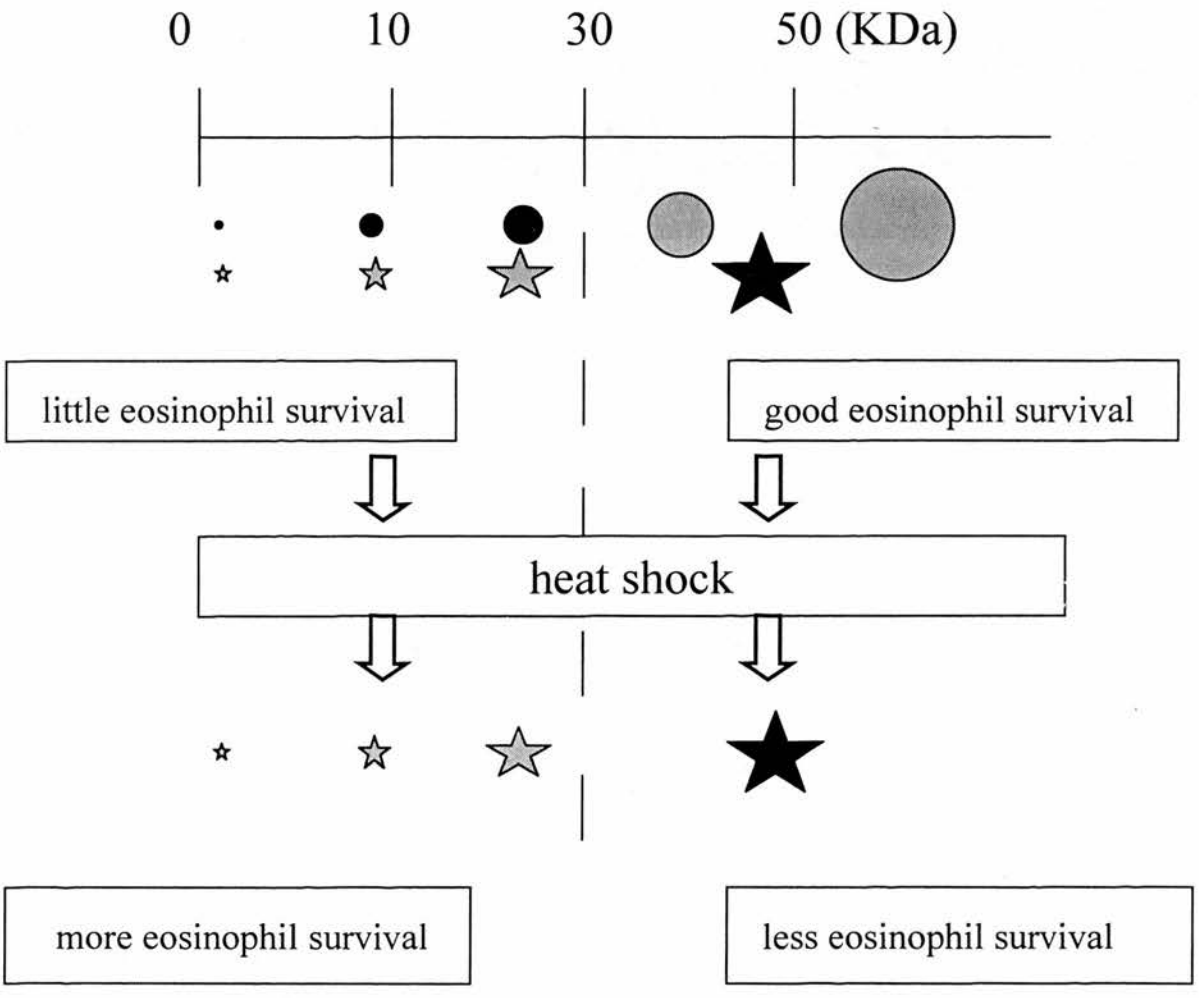


Figure 6-12; The effect of incubation with supernatant of L540, which had been separated according to molecular mass and heat shocked. Purified eosinophils (3.0 x10^6 cells/ml) cultured in Iscove's DMEM containing 10% autologous serum were treated with 10% of supernatants from L540 HRS cell line in which the contents of the supernatant had been isolated according to molecular mass by the series of centrifugations. In addition to, those supernatants are heat treated from 40°C to 100°C for 15 minutes. 'NH' indicates as non-heated. 'L540' indicates the control condition which is whole L540 10% supernatant. Then eosinophil survival after 5 days or 10 days were morphologically assessed. All the values represent mean with standard error of three different donors.

Fractions bigger than 30KDa (30KDa<x<50KDa and x>50KDa) showed a very similar pattern to whole supernatant. Indeed, in 5 days culture, heat shock treatment did not affect any survival rates. However, in 10 days heat shock treatment actually reduced their survival effect. These indicate that the fractions in 30KDa<x<50KDa and x>50KDa contain some heat unstable substances. They may be such as peptides and proteins.

On the other hand in the fraction less than 30KDa (x<10KDa and 10KDa<x<30KDa) the pattern of the survival rate showed differently. In these cases, heat shock treatment actually improved the survival rates. This indicates that these fractions may contain heat stable survival factors, and heat unstable pro-apoptotic factors. After heat treatment, heat stable substances, for example, lipid, may be responsible for eosinophil survival. On the other hand, the heat unstable substance, for example protein, may be acting as an inhibitor of survival. However, it is likely that substances x is heat stable in this fraction.

Table 6-12 and figure 6-12-B summarise this experiment. It may be possible to conclude that substance x in the fraction smaller than 30KDa is a heat stable substance (e.g., lipid), but in fractions bigger than 30KDa is heat unstable substance (e.g., protein).



heat unstable substance,e.g. protein

heat stable substance,e.g. lipid

survival inducer

survival inhibitor

Figure 6-12-B; Summary of the experiment (6-2-11) molecular weight cut off and heat shock. Fractions bigger than 30KDa contain eosinophil survival inducers, which are heat unstable, such as protein, but smaller than 30KDa contains eosinophil survival inhibitor, which are heat stable, such as lipid.

days	heat	L540	x<10	10<x<30	30<x<50	x>50
5	stable	ic	**survival**	**survival**	ic	ic
	unstable	ic	inhibition	inhibition	ic	ic
10	stable	inhibition?		survival	?	?
	unstable	survival		inhibition	**survival?**	**survival**

Table 6-12; Summarised from the heat shock experiment (figure 6-12, 6-12-B). 'survival' indicates that they contain eosinophils survival factors. 'inhibition' indicates inhibition of eosinophil survival. 'ic' indicates that the results are inconclusive. Isolation of molecular mass is KDa.

Additionally, if chemokines exist in the supernatant of L540, they are in the fraction less than 10KDa. Heat treatment should disturb the structures and function of chemokines. In the fraction less than 10KDa, after the heat treatment, survival rate was in fact improved. Therefore, it is unlikely that chemokines are the eosinophil survival factors in the supernatant of L540.

Summary

- The fraction bigger than 30KDa may contain the eosinophil survival factors which are heat unstable, such as proteins and peptides.
- The fraction smaller than 30KDa may contain the eosinophil survival factors which are heat stable, such as lipids.
- The fraction smaller than 30KDa may contain the pro-apoptotic substances, which are heat unstable, such as proteins and peptides.

- It is unlikely that chemokines in the supernatant of L540 are the eosinophil survival factors.

6.13. Co-culture with eosinophils and L540 cells, phagocytosis by L540 cells

Previous experiments were focused on eosinophil survival by supernatants derived from HRS cell lines. This time to investigate the interaction between HRS cells and eosinophils further, in the following experiments, eosinophils were cultured with L540 cells.

When eosinophils and L540 cells were co-cultured together *in vitro*, there were interesting phenomena observed. They were 1) eosinophils were phagocytosed by L540 cells, 2) increased number of multinucleated L540 cells (see 6.14.), 3) disappearance of eosinophils or L540 cells, depending on the ratio of the cells in the culture (see 6.15.).

Phagocytosis activities by HRS cells have been occasionally reported (see introduction 6.1.14.), however, there are no reports of phagocytosis by the T cell derived L540 cells. During the co-culture with L540 and eosinophils, observed frequent phagocytosis or phagocytosis-like activities by L540 are shown in figure 6-13. Typically, L540 extended its 'hand' like structures to surround eosinophils for possible engulfment. This feature was more commonly observed when L540 became bigger and multinucleated (see 6.14., 6.15.).

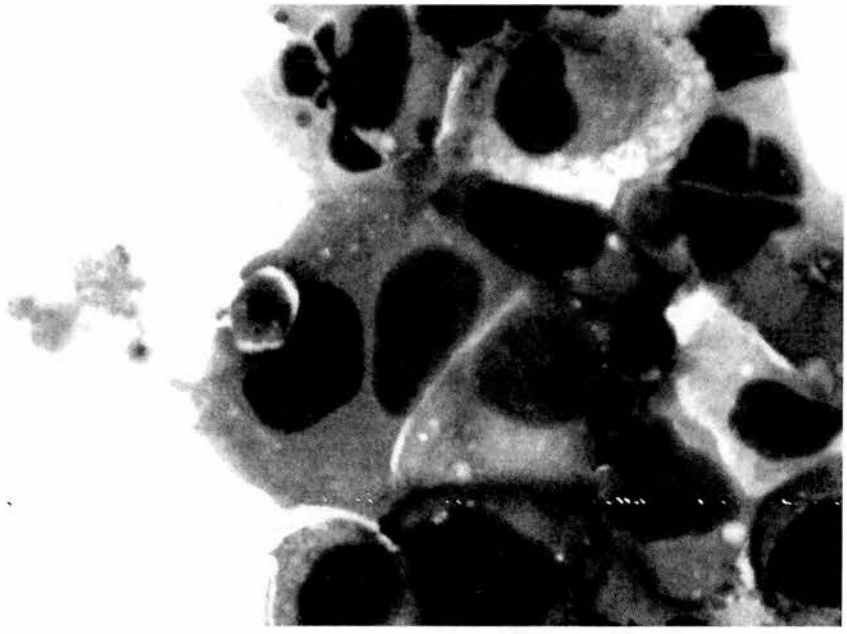


Figure 6-13; Phagocytosis of eosinophils by L540 cell. When eosinophils and L540 cells were co-cultured together, phagocytosis or -like phenomena were frequently observed. (magnification x 100)

This is the first report showing that L540 cells may have the ability to phagocytosis other cells.

Ingested eosinophils by L540 cells were both apoptotic and viable. The precise mechanism is unknown.

Summary

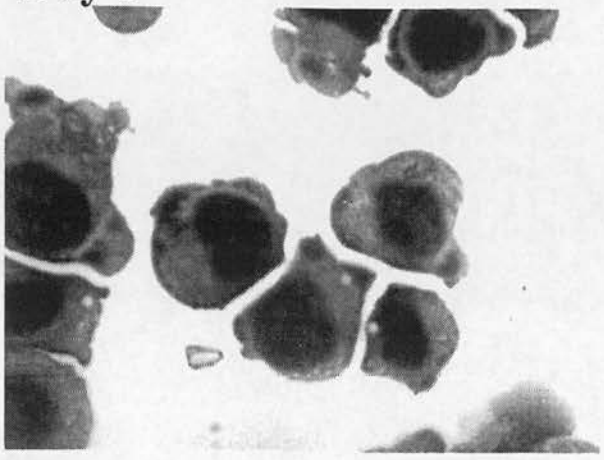
- L540 cells may phagocytose eosinophils during co-culture.
- Engulfed eosinophils were both viable and apoptotic.

6.14. Multinucleated HRS cells

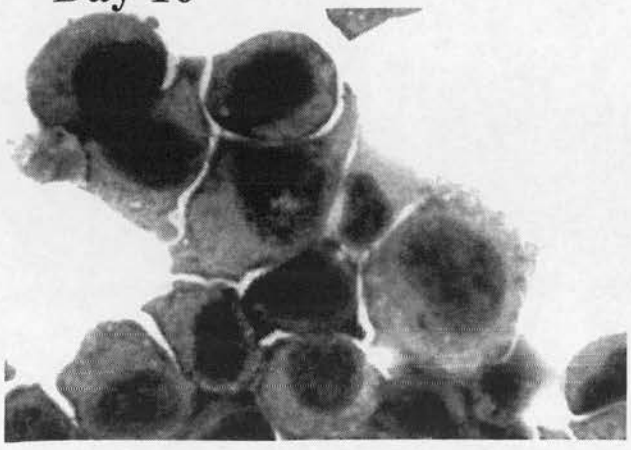
By definition, Hodgkin cells possess one nucleus per cell and Reed-Sternberg cells possess more than two nuclei per cell. In addition, multinucleated Reed-Sternberg cells are generally regarded as being a more aggressive and advanced form of cancerous cells. As shown in figure 6-1-2, isolated HRS cells from biopsy samples often are multinucleated with condensed chromatin like structures within the nuclei.

Usually in cell culture L540 are single nucleated cells, about 95% mono-nucleated, 5% multinucleated. As shown in figure 6-14-A, in non-stimulated L540 cells, most of the cells are single nucleated cells. (L540 cells have a T cell morphological phenotype.) When L540 cells were co-cultured with eosinophils, in 7-10 days culture, the number of multinuclear L540 increased (see figure 6-14-B for the graph). Those multinucleated cells possessed features similar to HRS cells obtained from biopsy samples and are typically referred to as 'owl eye' multinucleated cells.

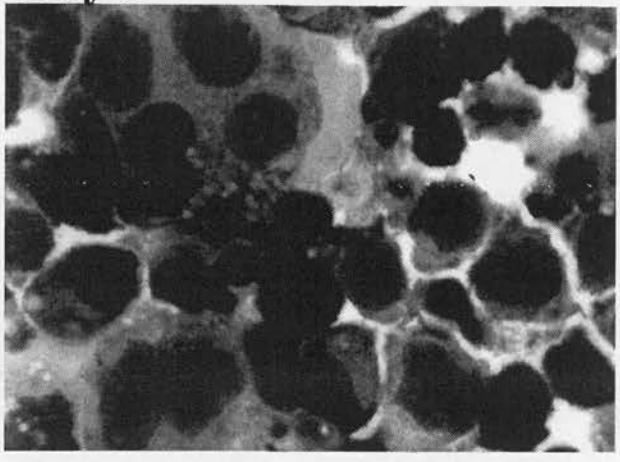
Day 0



Day 16



Day 9



Day 11

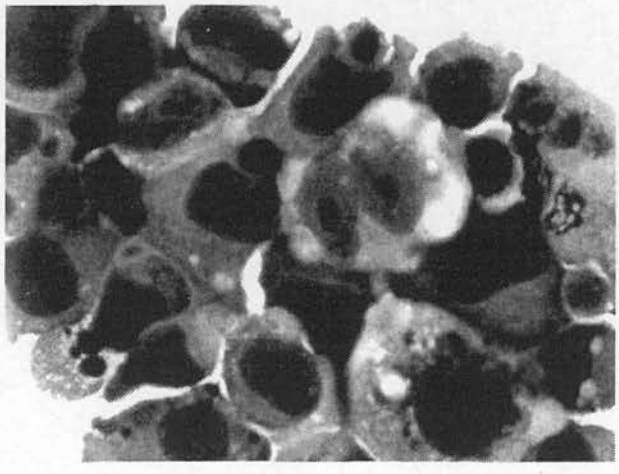


Figure 6-14-A; The turn over of HRS cells. HRS cells L540 were cultured with eosinophils for the time indicated. From single nucleated L540 at the resting stage (day 0), soon after co-cultured with eosinophils, the number of multi-nucleated cells were increased (day 9 and 11). After 16 days, the majority of HRS cells became the single nucleus cells, again. (Magnification x 100)

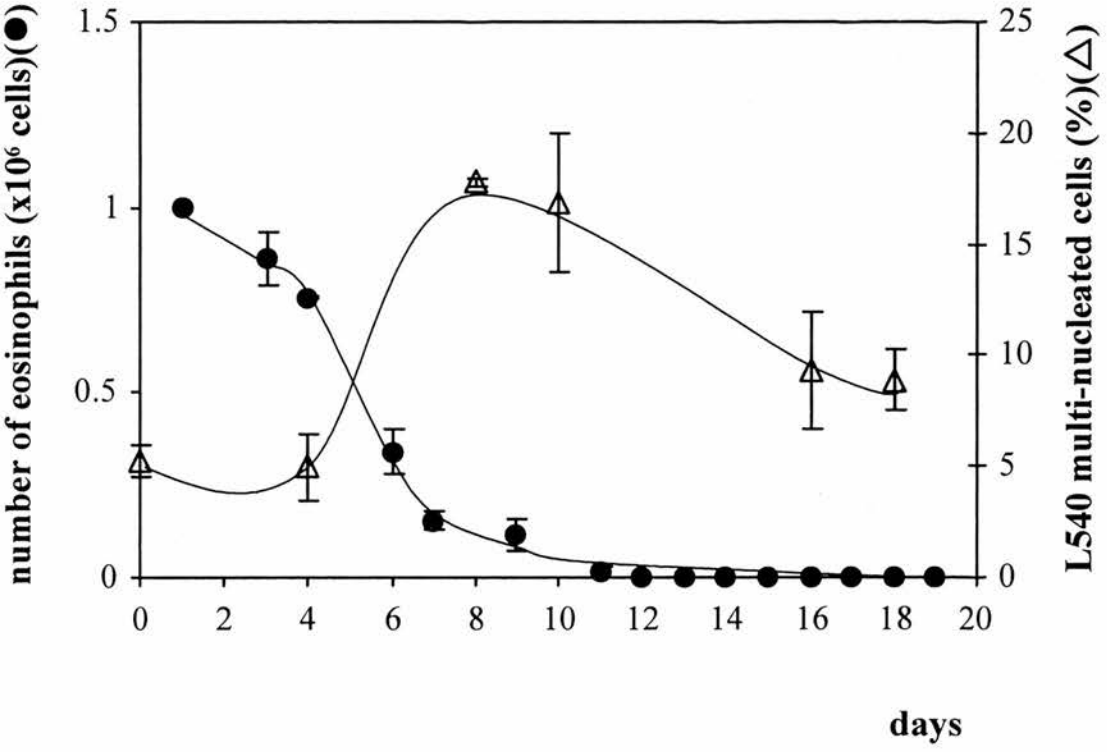


Figure 6-14-B; The relationship between eosinophil number and multi-nucleated L540 cells. Purified eosinophils (1.0 x 10^6 cells) were cultured with L540 cells in RPMI1640 containing 10% FCS and kept at 37 ° C for 16 days. The number of eosinophils (●) and multi-nuclei L540 cells (△)were counted. All the values represent mean with standard error of three experiments performed on the separate occasions. The number of multinucleated L540 cells increased between 8 and 10 days but decreases after the peak. In contrast eosinophils number kept decreasing and no eosinophils after 11 days.

However, after a certain period of culture the number of multinucleated cells decreased and eventually went back to the basal levels.

To investigate if there was any correlation between eosinophils and multinucleated cells of L540, the number of both of them were counted and the results are shown in figure 6-14-B. Eosinophil numbers steadily declined during co-culturing with L540. In contrast the number of multinucleated L540 cells increased at between day 8 and 10 and then decreased after this peak.

The mechanism regulating the ability of those cells to become multinucleated cells, which is believed to be the more aggressive form of cancerous cells, is currently unknown. However, it may be that some stimuli liberated by eosinophils change the appearance of L540 cells.

To further elucidate the causes of multinucleated cells by eosinophils, as the next experiment investigated, eosinophils may be added constantly or after an interval of certain periods to the L540 cell culture to observe the correlation between the number of those cells in the cell culture.

The same experiment was also performed on eosinophils and Jurkat cells, which are T cell leukaemia cells. No increase in the number of multinucleated cells were observed (data not shown).

Summary

- Multinucleated L540 cell numbers increased about 8-10 days after co-culture with eosinophils.
- Once eosinophils disappear from the culture, the number of multinucleated L540 cells declined.

6.15. Ratio study

As it has been shown in 6.14., the increase in multinucleated cell number may be directly influenced by eosinophils. To investigate the relation between eosinophils and L540 cells further, differing numbers and ratios of eosinophils and L540 cells were co-cultured for up to 7 days. Eosinophils were added to L540 cells, the same number (eosinophils: L540 cells = 1:1) or 10 times L540 cells (eosinophils: L540 cells = 10:1) were examined. The results are shown in figure 6-15-A.

After 3 days incubation, one of the obvious features was that eosinophils and L540 cells attached to each other. After 7 days of incubation, the effects of the different ratios of the cells was striking. The ratio of eosinophils: L540 cells =1:1 (figure 6-15-A), after 7 days, eosinophils disappeared from the culture (figure 6-15-A shows 3, 6 and 10days). In contrast, eosinophils: L540 cells =10:1 (figure 6-15-A-2) culture showed no L540 cells but only eosinophils survived. The reasons for disappearance of eosinophils or L540 cells are not clear. However, it may be due to phagocytosis or 'uptake' of eosinophils into L540 cells. On the other hand, eosinophils might exhibit on ability for killing as they do for parasitic invaders. This will be discussed further in discussion 6.16.5.

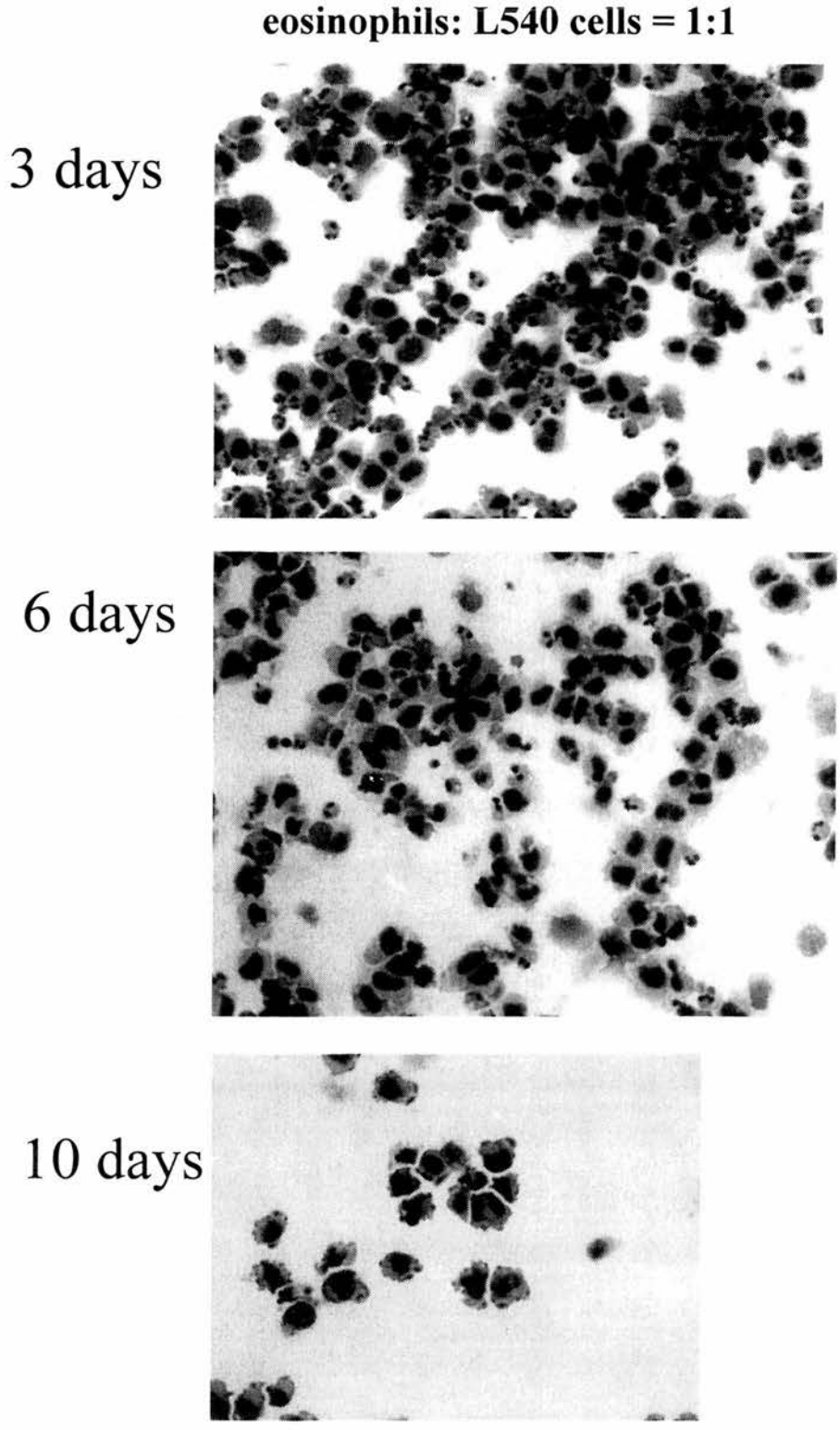
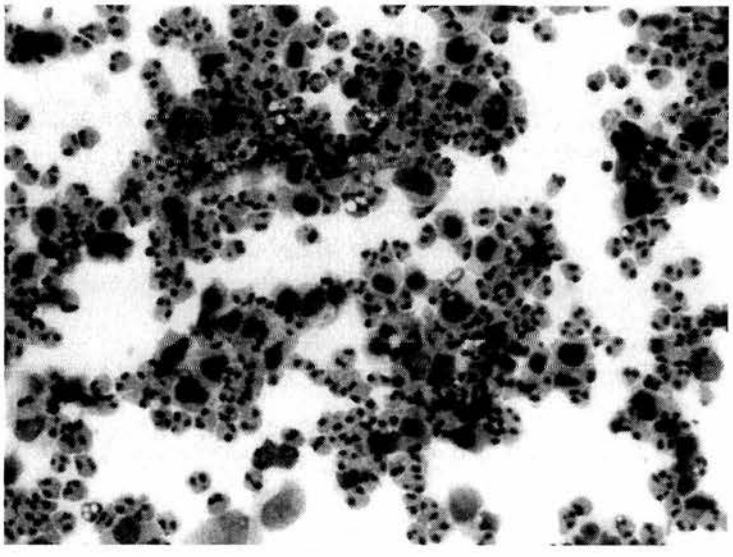


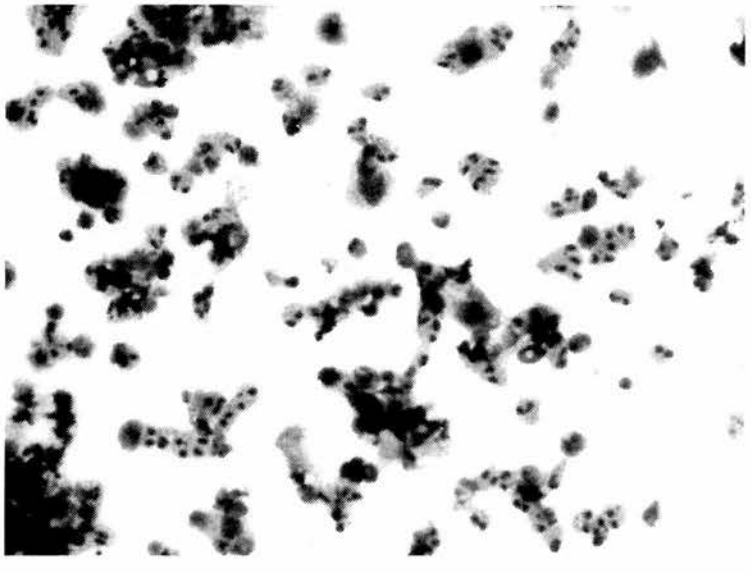
Figure 6-15-A-1; Co-culture of eosinophils and L540. Eosinophils and L540 cells were co-cultured at a 1:1 ratio for the time indicated. Eosinophils were gradually disappeared but L540 cells survived on day 10. Magnification x 63.

eosinophils: L540 cells = 10:1

3 days



6 days



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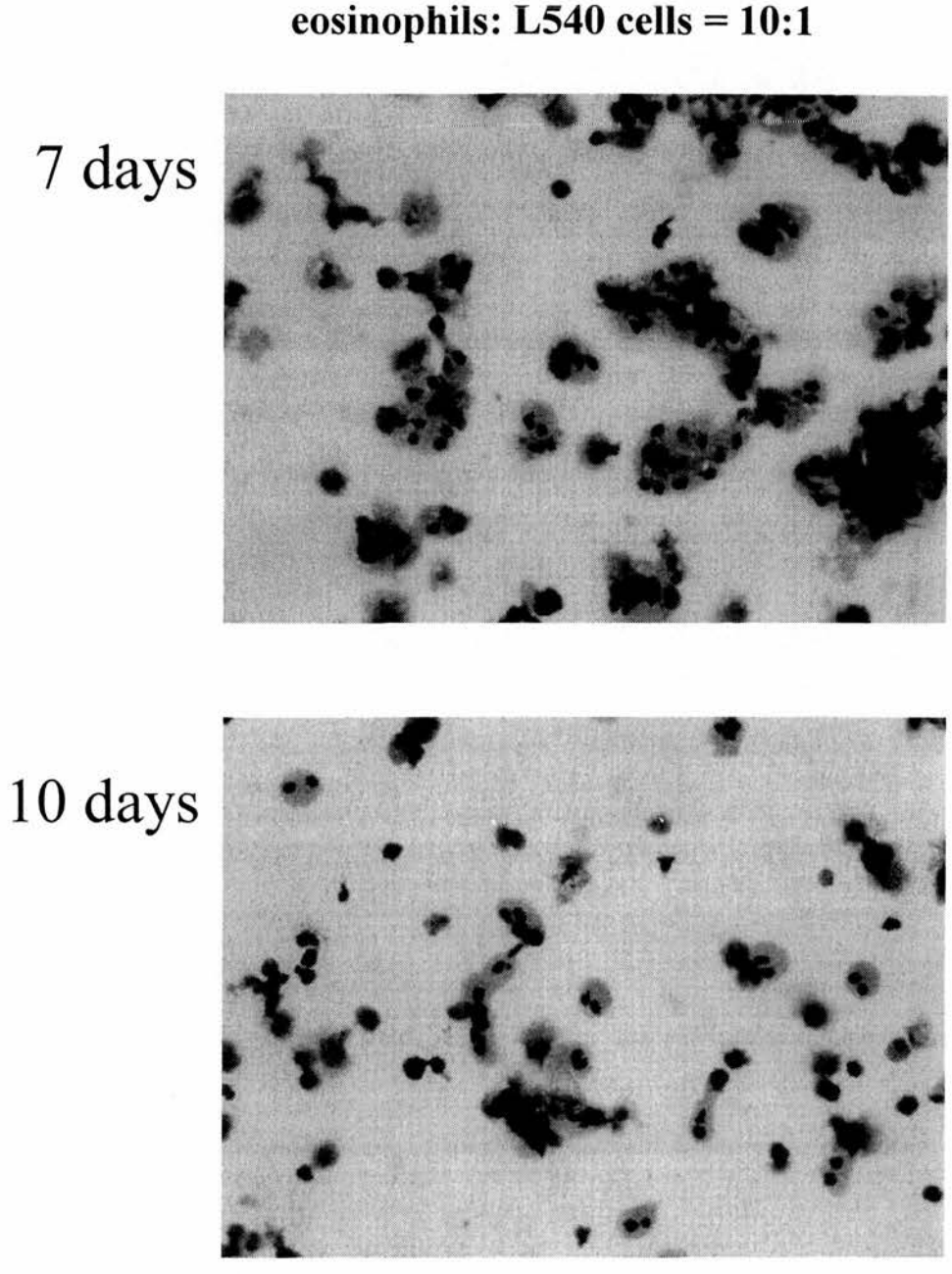

Figure 6-15-A-2; Co-culture of eosinophils and L540. Eosinophils and L540 cells were co-cultured at a 10:1 ratio for the time indicated. L540 cells were gradually disappeared but eosinophils survived on day 10. Magnification x 63.

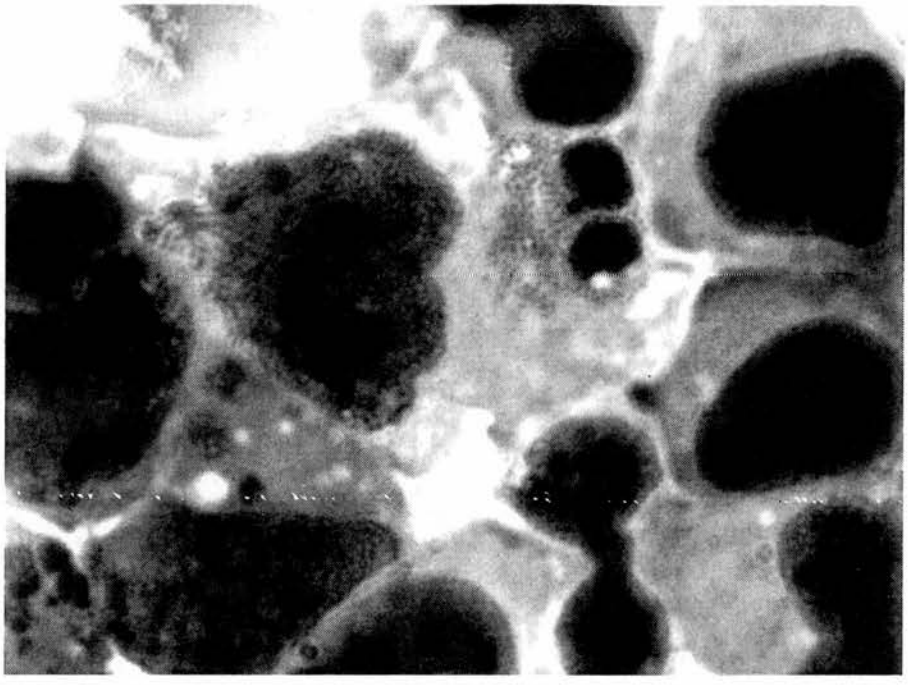

Figure 6-15-B; Interaction between eosinophils and L540 cells.
Eosinophils and L540 cells were co-cultured for 5 days. Eosinophil granules are seen in the cytoplasm of L540 cells. (Magnification x 100)

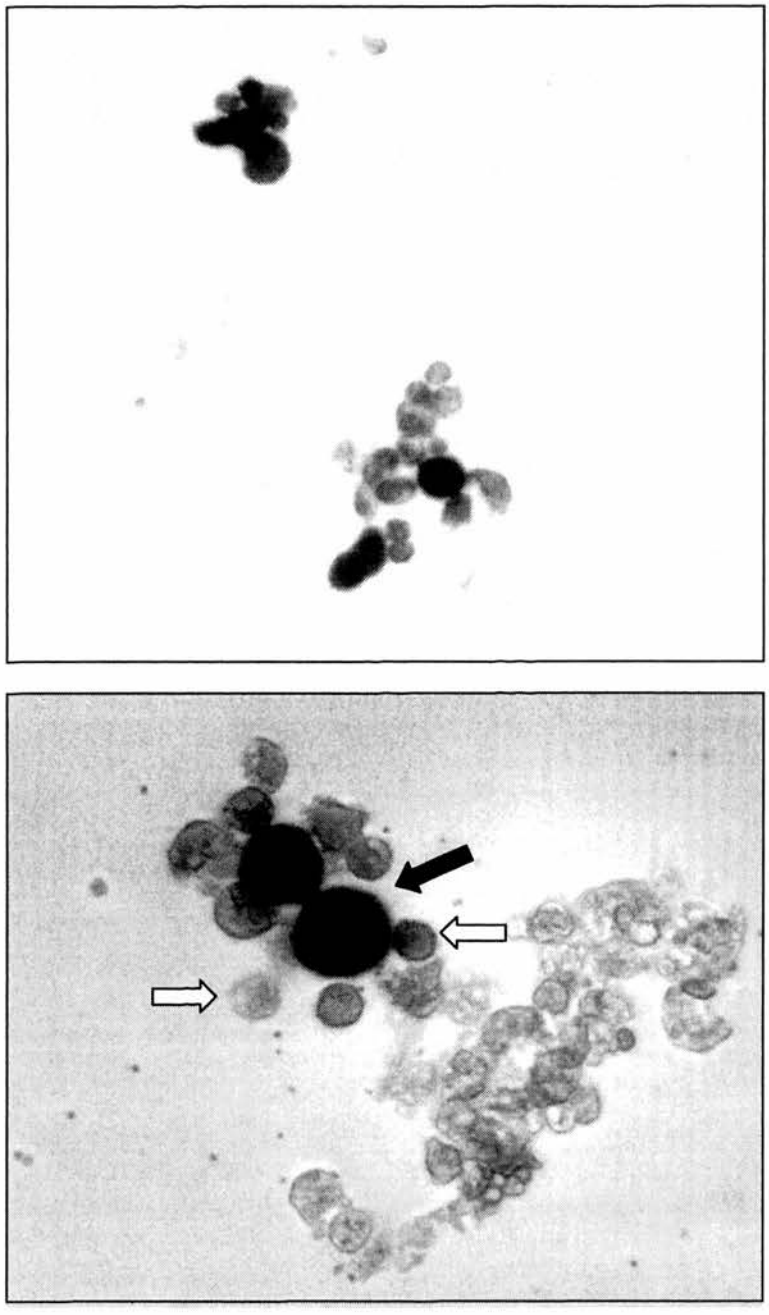

Figure 6-15-C; Co-cultured L540 cells and eosinophils are positively transfected by Ad-βgal. L540 cells (indicated as black arrow) and eosinophils (white arrow) were co-cultured together for 2 days and transfected with Adeno-βgal for 6 hours. Eosinophils attached to L540 cells were successfully transfected and showed βgal stained. (Magnification x 32 panel above, x 63 panel below.)

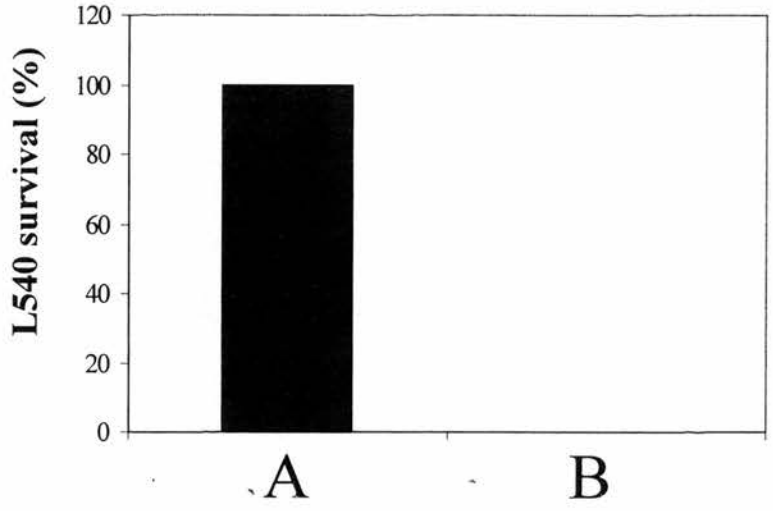

Figure 6-15-D; Effect of supernatant from L-540-supernatant-treated eosinophil and supernatant from L540 HRS cell line (B) on L540 survival. L540 (3.0 x 10^6 cells/ml) cultured in RPMI 1640 containing 10% FCS serum were treated with 10% of supernatant from L-540-supernatant-treated eosinophil (B) or without (A) and kept at 37°C for 5 days. After 5 days cell survival was assessed by trypan blue.

To extend this point, experiments were performed to investigate whether the release of granules contents from eosinophils may act to inhibit L540 cell survival (figure 6-15-D). Eosinophils were incubated with supernatants of L540 for 7 days. Then, the supernatant of this L540-supernatant-treated eosinophils culture was taken and put back into L540 culture, where the original supernatant from L540 came from. After 5 days, all L540 cells died. These experiments indicate that some toxic or apoptosis inducing substances from eosinophils may cause L540 cell death.

The contact between cells were so close that they appear to have fused into one cell in some cases. Those cells are shown in figure 6-15-B. Strangely, in some cases, eosinophils granules (stained in orange colour) were seen in the cytoplasm of L540 cells, indicated as black arrows. These cells were prepared by cytospin centrifugation and may therefore be subject to artifactual interpretation.

Interestingly, when those 'fused' cells were transfected with Adeno virus-βgal (Ad-βgal), eosinophils, which cannot be transfected by Adeno virus due to a lack of receptors, were indeed successfully transfected. (This experiment was kindly done by Dr Sallenave, CIR lab, University of Edinburgh.) These results are shown in figure 6-15-C. The precise reason for Ad-βgal transfection of those eosinophils is not known. However, this may indicate that L540 cells and eosinophils may be partially fused. Further studies are however required to investigate this intriguing hypothesis.

The relation between eosinophils and L540 cells is very peculiar and to our knowledge has never been reported before. There are some physical interactions

between eosinophils, L540 cells and some substances released by eosinophils may have an ability to kill L540 cells. These are very interesting observations and many experiments have to be performed to investigate further.

Summary

- When the ratio of eosinophils: L540 cells =1:1, only L540 survived.
- When the ratio of eosinophils: L540 cells =10:1, only eosinophils survived.
- Eosinophils and L540 cells may be fused and share the cellular features.
- L540-supernatant-mediated eosinophils may release substances, that can kill L540 cells.

6.16. Discussion

The studies herein have sought to elucidate the effects of eosinophilia and neutrophilia by HRS cells. The results presented in this chapter are unique and may be the first steps to understand eosinophil recruitment and survival at tumours of HD.

6.16.1. The substance x in L540 make eosinophils prolong their longevity.

Supernatants of L540 and L428 exerted for the first time a striking effect on both eosinophil and neutrophil survival. It is clear that there are strong eosinophil and neutrophil survival factors, substance x, in those supernatants. This substance x could be a known or an unknown factor or a cocktail of different factors.

However, from the experiments where supernatant of L540 was separated by molecular mass and exposed to different temperatures (6.10. to 6.12.), some interesting findings were made. (See also figure 6-12-B for summary.) Substances bigger than 30KDa and heat unstable, such as cytokines, proteins, etc., are likely to be the eosinophil survival factors. In contrast, substances smaller than 30KDa and heat stable, such as lipid and other low molecular weight substances may be the survival factors. From these results, substance x may be a cocktail of different substances of a various molecular masses and nature. Interestingly, substances less than 10KDa and heat unstable, such as peptides and small proteins, may be inhibitory factors for eosinophil survival. This might also include certain chemokines. Chemokines are known as the powerful eosinophil chemoattractants and survival factors, however, they can be ruled out from the list of candidates of eosinophils survival factors in these studies. On the contrary cytokines with

molecular mass bigger than 30KDa, may be candidates as survival factors. To investigate what cytokines are in the supernatants of L540, a series of targeted ELISA may be the direct way to determine. Although as shown in the table 6-1-7-B, there have been reports showing the production of various cytokines and chemokines from HRS cells and clinical samples. However, the list is still incomplete due to the lack of consistency of the methodology used to determine only selected cytokines in selected cell lines and clinical samples. A complete study, has to be performed to measure the amounts of all the known cytokines from all known or major HRS cell lines.

During this project, we chose not to directly investigate possible candidates for substance x. Thus, ELISA or other methods to detect possible candidates for substance x were purposely avoided due to the existence of too many different cytokines likely present in the supernatants from HRS cell lines. However, if they had performed, it would not even identify a whole substance x if substance x was yet an unidentified factors. In addition, these experiments are time consuming and expensive. Thus, our strategy was to leave screening programme, kindly offered by Dr Stuart Farrow at Glaxo SmithKline for the further investigation. Currently, all the supernatants from HRS cell lines are being investigated by screening of the known cytokines and chemokines at the protein level. Purification would be the next possibility to identity substance x. The challenge would be to ascertain the identity of novel substances involved in eosinophil survival. Unfortunately, the answers of those experiments have to be waiting for the future studies.

IL-5 and GM-CSF are two of the strongest eosinophil activators and survival factors known. In addition, in figure 6-5, L540 supernatant showed a slightly better effect at causing eosinophil survival compared to IL-5 and GM-CSF. The question has arisen whether supernatant of L540 may contain IL-5 and GM-CSF. To take this step further, neutralising experiments using anti IL-5 and anti-GM-CSF antibodies were performed to abrogate the survival effect of supernatant of L540. Anti-GM-CSF antibody 1.0μg/ml, which should be sufficient to neutralise 50% of the bioactivity of 0.5ng/ml rhGM-CSF (according to the manufacture) was used. (Similar dose-response to anti IL-5 antibody.) However, there was no effect on inhibition of eosinophil survival (figure 6-5). These could be because 1) no IL-5 and GM-CSF were present, 2) there was not enough antibody to neutralise. 3) There are other substances which may have a profound eosinophil survival effect. For further investigation, ELISA is required to measure accurately the amount of the IL-5 and GM-CSF in the supernatant of L540. If detected, a suitable amount of antibody should be determined for using in the neutralising experiments.

In addition, although IL-5 and GM-CSF are known as profound eosinophil survival factors, however, in long term culture its effect may not be as strong as the supernatant of L540. Eosinophils treated with L540 supernatant were occasionally observed surviving up to about 30 days in the cell culture sometimes even without changing the medium. IL-5 and GM-CSF are unlikely to produce such striking results. This indicates that there may be other substances in the supernatant of L540 that mediate the powerful eosinophil survival effect observed in this study.

6.16.2. The role of NF-κB in mediating eosinophil survival by HRS cells

NF-κB is an important regulator of eosinophil fate. In this chapter it is shown that various NF-κB inhibitors abrogated L540-supernatant-mediated eosinophil survival, suggesting that NF-κB is indeed playing a role in L540-supernatant-mediated eosinophil survival (figure 6-8 and 6-9). There may be some substances in the supernatant of L540 that may be pro-apoptotic which may be enhanced by co-incubation with NF-κB inhibitors. TNFα is a likely candidate since the presence of TNFα has been previously detected in L540 (Foss *et al.*, 1993).

To investigate the role of NF-κB further, other studies to determine the level of NF-κB activation should be examined, for example, measuring the expression of IκBα and p65 by Western blotting, p65 translocation by immunofluorescence studies etc. Not only NF-κB but other pathways, such as JAK, STATS, MAPK etc., should also be investigated in the next stage, especially, substance x might trigger various signalling pathways in the cells.

NF-κB is a potential target transcription factor for cancer therapy. In HD tumours, there may be excessive NF-κB activation occurring in various cells. For example high constitutive activation of NF-κB in HRS cells (Cabanne *et al.*, 1999 and Krapmann *et al.*, 1999) results in the high metabolic activities in those cells. Similarly, as shown here, L540-supernatant-mediated eosinophil survival is dependent on the NF-κB pathway. NF-κB activation not only regulates survival, but also may be pro-inflammatory due to the production of pro-inflammatory cytokines. Consequently production and release of cytokines may cause further progression of

the tumour. The supernatant of L540 and other HRS cell lines do contain some survival factors for eosinophils and neutrophils. Interestingly, Fabineen Allie at the University of St Andrews has found that lymphotoxin in the supernatants of HRS cell lines does activate NF-κB in HeLa cells. However, lymphotoxin did not directly induce eosinophil survival, but may activate in other types of cells at the tumour site.

In general, the most drugs used in chemotherapy inhibit NF-κB activation. For example, corticosteroid Prednisone, is the standard drug used for the treatment of HD. Steroids are believed to inhibit NF-κB transcription activities binding to DNA (Adcock *et al.*, 1999).

6.16.3. Neutrophils and T cells in HD

The number of neutrophils accumulated at tumours of HD is limited, thus, fewer studies have been done on neutrophils. There are yet no conclusive reasons given as to the mechanism and function of neutophil accumulation. Eosinophils and neutrophils arise from a common precursor in bone marrow and share many common signalling pathways, including NF-κB. Similar to eosinophils, it has been shown in this chapter that supernatant from L540 exert its effect on neutrophil survival. Therefore, it is reasonable to assume that in the supernatant of L540 there are common survival factors, which may influence the NF-κB pathway. On the contrary, there are some differences in activation pathways and their activator ligands between neutrophils and eosinophils. It would be interesting to perform the

comparison studies of molecular weight cut off and heat shock experiment in neutrophils as has been done in eosinophils (figure 6-12).

Other types of cells, e.g. T cells, fibroblasts, macrophage, etc., should be considered for the further investigation. For example, Th2 cells are highly expressed in HD and may contribute to the recruitment of inflammatory cells (Poppema *et al.*, 1998). In addition, the involvement of surrounding tissue cells, such as fibroblasts, may be one of the causes of recruitment of inflammatory cells. Especially since fibroblasts have the capability to release eotaxin (Teruya-Feldstein *et al.*, 1999) which is likely one of the direct causes of eosinophil recruitment. To understand eosinophilia further, other types of cells should be included for further studies in order to elucidate the mechanism and function of eosinophilia at the HD tumour.

6.16.4. Further studies

To investigate the function and mechanism of eosinophilia and accumulation of other inflammatory cells further, the following experiments should be considered.

Activation

Since eosinophil and neutrophil survival has been shown, it would be worth performing, activation and migration studies. To investigate if HRS cells activate eosinophils, extracellular release of granules products may be measured as an indicator of activation. This could be measured by evaluation of eosinophil peroxidase or MBP levels, which are typical markers of eosinophil degranuelation.

In addition, preliminary experiments to determine the possibility of release of cytotoxic granules contents of eosinophils was performed. (Data not shown, but also see 6.15. for the ratio studies. Briefly, eosinophils were cultured with the 10% supernatant of L540 for 7 days. The supernatant from which eosinophils were cultured was taken and put back onto the L540 cell line culture for the total volume of 10%. After 24 hours, all L540 cells were killed.) This experiment strongly suggests the existence of cytotoxic substances in the supernatant of eosinophils culture which had been treated with L540 supernatant. The precise identity of these substances is unknown, but it is highly likely that they are the contents of cytotoxic granules. To determine this possibility, extracellular release of granule products should be measured.

In addition, as for eosinophil activation the influence of other inflammatory cells such as T cells should be examined. Activated inflammatory cells are likely to influence other inflammatory cells. Thus, activation studies are very important to investigate the roles and mechanism of accumulation of inflammatory cells at the site of HD tumour.

Migration

Migration study would be important to investigate the mechanism of accumulation of inflammatory cells at the HD tumour. In the study of asthma, eosinophil migration studies have been investigated both *in vivo* and *vitro*. Therefore, the knowledge from migration studies of eosinophils in the airways could be applied to HD study. These can be done with eosinophils and HRS cell lines, but also, it would

be interesting to see the influence of other types of cells such as fibroblasts to cause eosinophil migration as it has been shown that eotaxin is released from fibroblasts (Teruya-Feldstein *et al.*, 1999).

An interesting study by Schell-Frederick *et al.*, (1988) showed that a subfamily of the L428 cell line, L428 KSA, inhibits neutrophil migration. However, this study was limited since only this subgroup of cell line and neutrophils was examined. It was concluded that this could be the reason for the immunodeficiency observed in HD. However, the neutrophilia in the tumour of HD was not discussed. Additionally migration studies using eosinophils as well as neutrophils and T cells should be performed using other HRS cell lines.

Galectin-9

Human galectin-9 was first cloned from the cDNA library derived from HD (Türeci *et al.*, 1997), and detected in the sera from 10 of 18 patients with HD (Sahin *et al.*, 1995). Galectin-9 is a family of thiol-dependent lectins with a conserved carbohydrate recognition domain and high β-galactoside binding activity. It regulates intra-cellular binding resulting in the promotion of cell proliferation or apoptosis. Galectin-9 is highly expressed in HRS cells and Jurkat cells, but not THP-1 and monocytes. Recently sequence studies revealed that galactin-9 was identified as ecalectin (Matstumoto *et al.*, 1998), which has high eosinophil chemoattractant properties. Therefore, galectin-9 is good candidate for causing eosinophil migration in the HD tumours. Eosinophil migration assays can be performed to investigate the effect of galectin-9. (This experiment idea was kindly suggested by Prof R Jarrett, University of Glasgow.)

6.16.5. Cell-cell interaction in HD

The direct relationship between HRS cells and eosinophils are currently unknown. From the observation studies shown in this chapter, when eosinophils and L540 cells were co-cultured, the following observations were made; 1) L540 cells may have the ability to phagocytose, 2) increased number of multinucleated cells 3) L540 cells may be killed by eosinophils depending on the ratio of eosinophils and L540 cells.

As previously reported, HRS cells are capable of emperipoiesis and phagocytosis (see introduction 6.1.14. for summary). In addition, the observations from this thesis reveal that there may be some phagocytosis or emperipoiesis activities by L540 cells. It is still unclear why L540 cells which are derived from a T cell origin are capable of phagocytosis. When another T cell origin cell line, Jurkat cells were co-cultured with eosinophils, there was no phagocytosis observed. Thus, phagocytosis or emperipoiesis may be one of the features of HRS cells despite their origins. Recently studies have revealed that not only monocytes, but also other types of cells can indeed phagocytose other cells. For example, epithelial cells in the airways engulf apoptotic eosinophils (Walsh *et al.*, 1999). There might be some feature changes in those cells allowing them to phagocytose.

Another interesting observation was that when L540 cells and eosinophils were co-cultured, occasionally eosinophil granules-like structures, which are stained orange by eosin, were observed in the cytoplasm of L540 cells. However, to provide

definite proof for this phenomenon, electron microscopic observation is required. However, if it is true that granules are indeed in the cytoplasm of L540 cells this may be further evidence for the engulfment of eosinophils. However, whatever the reasons, if granules are indeed in the cytoplasm of L540 cells, toxic proteases from granules would be deleterious for L540 cells. This may be the explanation why some L540 cells were killed by eosinophils, when the eosinophil ratio was high against L540 cells. On the other hand, this might disturb the normal intracellular activities of HRS cells, such as mitosis. This may be the reason why there was an increased number of multinucleated cells when L540 cells were incubated with eosinophils.

In normal circumstances, only apoptotic eosinophils are phagocytosed. In those cases, once cells become apoptotic, granules are still present in the apoptotic body. In case of L540, it was observed that they engulf any eosinophil, dead or alive. So it could be possible that L540 may ' emperipoiesis ' live eosinophils rather than phagocytose apoptotic eosinophils, and the results in the existence of granules from eosinophils in the cytoplasm of L540 cells. Or it may be possible that eosinophils are thought to be 'viable' might be at an early stage of apoptosis without obvious morphological changes, such as chromatin condensation. To determine the phagocytosis or emperipoiesis activities more specifically, other methods have to be introduced, for example, electron microscopy, for the future studies.

When L540 cells and eosinophils were incubated together, the number of multinucleated cells was increased (see figure 6-14-A and -B). Those multinucleated cells are very like Reed-Sternberg cells in the biopsy samples (see

figure 6-1-2). The observations made here suggesting that multinucleated cells were indeed peculiar and abnormal looking. Nuclei are usually in two, four or five per cell. Very often nuclei appear paired because they are symmetrical, which are identical shapes and patterns of darkly stained areas. This may suggest that those cells may have failed for the normal cell mitosis but fused into one cell after nuclei are divided. Therefore, they do contain identical looking nuclei in the same cell. The reasons for this observation are unknown. As it has been mentioned above, it is possible that eosinophil granules may be disturbing some normal intracellular functions to cause multinucleated cells. These interesting observations however require further experiments.

Additionally, in figure 6-16-5, the biggest L540 cells observed during this project is shown. Compared to surrounding eosinophils and the number of the nuclei present, this phenomenal cell may be the result of the failures of normal cellular activities.

6.16.6. Is eosinophilia beneficial or deleterious for the pathogenesis of HD?

These studies have showed that there may be a role for eosinophils in the pathogenesis of HD. What roles eosinophils may be playing in terms of the progress of the disease seems unclear. Especially co-culture experiments raises the questions of the functions of eosinophils. Incubation with eosinophils increased multinucleated HRS cells, which are regarded in the clinical situation as a more aggressive form of the tumour. On the other hand, excess amounts of eosinophils do kill L540 cells. This might indicate that eosinophils could be beneficial for the removal of HRS cells.

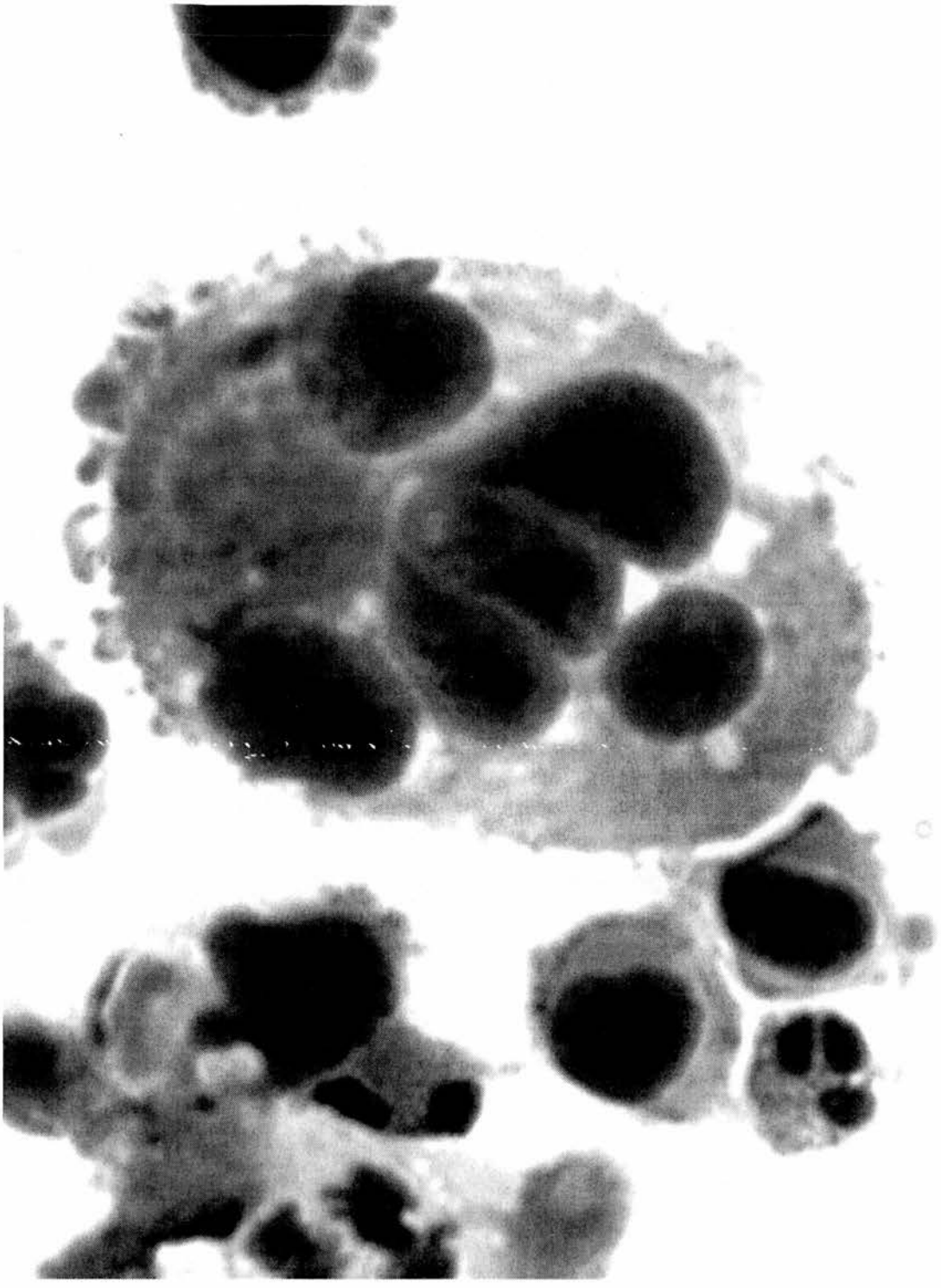

Figure 6-16; Giant L540 cells. Eosinophils and L540 cells were co-cultured for 5 days. Multi- nucleated giant cell is seen. (Magnification x 100)

This debate can be applied to other types of cancer studies and eosinophilia. As it has been reported that in the pathogenesis of cancer, eosinophilia could be beneficial or deleterious for the host (also see 6.1.13. for eosinophils and other cancers). There have not yet been enough studies to determine the role of eosinophils in cancer. Nevertheless, it may depend on the type of cancer. However, it is likely that in HD, eosinophils are not favourable for prognosis. Therefore, the removal of eosinophils from HRS cells may be still a favourable therapeutic target.

HD and inflammation are very different, yet, share some similarities in terms of accumulation of inflammatory cells. If eosinophil accumulation in HD is undesirable, inflammatory diseases such as asthma share a similar therapeutic target to remove excess eosinophils

Chapter 7
General conclusions

7.1. General conclusion

Accumulation of eosinophils is one of the features of inflammation but eosinophilia is also seen in other diseases (shown in table 7-1). As has been discussed in chapter 6, although HD and inflammation diseases are diverse and result in different pathogenesis, they may share the common characteristic feature, eosinophilia, and the therapeutic targets.

Allergic Disorders	***Neoplastic and myeloproliferative diseases***
bronchial asthma	Hodgkin's disease
allergic rhinitis	T cell lympyhoma
onchocercal dermatitis	hypereosinophilic syndrome
atopic dermatitis	bronchogenic carcinoma
drug reactions	
autoimmune reactions	***Vasulitic granulomatous diseases***
multiple sclerosis	temporal vasculitis
graft refection	Chung-Straus syndrome
	Polarteritis
Infectious parasitic diseases	Wegner's granulomatosis
toxocariasis	
filariasis	***Others***
schistosomiasis	HIV
trichinosis	

Table 7-1; The diseases associated with eosinophilia (modified from Krogel *et al.*, 1994)

Activated eosinophils are deleterious to host tissue due to the release of pro-inflammatory cytokines (e.g., IL-8) and histotoxic contents resulting in further progress of the disease. Therefore, the removal of eosinophils is beneficial for preventing tissue damage. For the 'silent' removal of eosinophils, eosinophil

apoptosis is essential in order to inhibit their activities and being phagocytosed by macrophages and other types of cells.

Eosinophil survival and apoptosis pathways are complex but at least NF-κB pathway seems to play an important role. In this thesis, it has been shown that inhibition of NF-κB is important for enhancing eosinophil apoptosis. Gliotoxin and Mg132 inhibit NF-κB and induced significant eosinophil apoptosis especially when stimulated with TNFα. Although TAT-IκBα showed a significant effect on eosinophil apoptosis, this effect was however donor dependent. In those donors whose eosinophils did not respond to TAT-IκBα, NF-κB activation may have already occured. During the immunofluorescent staining of p65, it was notable that eosinophils from a few donors had p65 translocation in the nucleus without any stimuli, whereas normally p65 was observed in the cytoplasm from other donors. This suggests that in some donors, the NF-κB pathway is highly activated and may result in the different levels of response towards NF-κB inhibitors. Secondly, the mechanism of TAT-IκBα, gliotoxin and Mg132 on inhibition of NF-κB is different which may have different influences in different donors. Some inhibitors may disturb other signalling pathways but not necessary specifically only the NF-κB pathway. However, although inhibition of NF-κB is still one of the key issues in the induction of eosinophil apoptosis future investigations should consider other intracelluar pathways.

Table 7-2 shows the potential therapeutic anti-eosinophil drugs and reagents. Mainly, their functions act to suppress eosinophil recruitment, activation and survival. Some (e.g., corticosteroids) may work on inhibition of NF-κB pathway

(Adcock *et al.*, 1999), but others (e.g., IL-5, IL-3, GM-CSF antagonists) may do so on tyrosine cascades.

Table 7-2; Potential anti-eosinophil reagents for the clearance of accumulated eosinophils at inflammatory sites. (Modified from Sampson, 2000.)

Corticosteroids
IL-10R agonists or IL-10
Leukotriene modifiers
IL-5, IL-3, GM-CSF antagonists
Eotaxin, RANTES antagonists
IL-4, IL-13 antagonists
VCAM, VLA-4 antagonists
Anti-IgE agents
CCR3 antagonists

For example, corticosteroids are known to induce eosinophil apoptosis both *in vitro* and *in vivo.* As has been shown in asthma patients, corticosteroids enhance the clearance of apoptotic eosinophils by macrophage phagocytosis in airways (Woolley *et al.*, 1996). However, eotaxin and RANTES antagonists may be necessary to inhibit further eosinophil recruitment at inflammatory sites. IL-10 (Takanashi *et al.,* 1994) has been shown to inhibit LPS induced eosinophil survival. Woolley *et al.*, (1994) has demonstrated that the inhibition of eosinophilia in sensitised Brown Norway rats by administration of IL-10. BION-1 is a monoclonal antibody against the beta(c) of IL-5, IL-3, GM-CSF receptor (Sun *et al.,* 1999) and has been shown to inhibit eosinophil survival. ICAM-1, VCAM-1, β2 integrin (Chihara *et al.*, 2000) expressed on epithelial cells, activates eosinophils, therefore, VCAM antagonist acts to inhibit eosinophil activation and accumulation.

These reagents may prevent eosinophil recruitment at inflammatory sites, therefore, eosinophilia may be suppressed. However, to resolve inflammation, there are other issues that have to be targeted, such as suppression of recruitment of inflammatory cells, lower cellular activities of inflammatory cells and induction of apoptotic inflammatory cells. For those reasons, generally at inflammatory sites, cellular metabolisms have to be suppressed in various cells. NF-κB is one of the key transcription factors responsible for pro-inflammatory consequences in various types of cells. Therefore, NF-κB inhibitors are often used as anti-inflammatory drugs, for example salicylates (Yin *et al.,* 1998) and Helenalin (Lyss *et al*, 1997). Inhibition of NF-κB at the inflammatory sites exerts its effect not only by suppressing transcritiptional activity but may also induce apoptosis in accumulated inflammatory cells.

As previously introduced in this thesis, HIV-1 TAT may be a promising peptide for future therapeutic purposes. TAT can be transduced into almost any type of cell, resulting in the transduction in various types of cell at the same time. For example, TAT-IκBα may be useful in suppressing cellular activities in different cell types at the inflammatory sites. This might prevent the pathogenesis of inflammation.

On the other hand, if TAT can be transduced into specific cell types, it would be a strong advantage, too, especially *in vivo*. The mechanism of transduction system of TAT is not fully elucidated. However, in the future, it may become possible to 'design' a TAT peptide for transduction into the selective cell types. This might enhance the ability of TAT and its therapeutic benefit *in vivo*.

For therapeutic purposes, the mechanisms regulating inflammation have to be understood in more detail.

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Appendix

Inhibition of nuclear factor-κB activation un-masks the ability of TNF-α to induce human eosinophil apoptosis

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Apoptosis renders eosinophils functionally effete and marks them for 'silent' removal from inflamed sites by macrophages. We show, for the first time, that eosinophils exposed to TNF-α rapidly lose their cytoplasmic levels of IκBα, the inhibitory subunit of NF-κB. Consequently, TNF-α triggers NF-κB mobilization from the cytoplasm to the nucleus, as determined by tracking the NF-κB subunit p65 by immunofluorescence and Western blot analysis. Inhibition of TNF-α-mediated IκBα degradation and NF-κB activation by gliotoxin or the proteasome inhibitor MG-132 un-masks the caspase-dependent pro-apoptotic properties of TNF-α. In addition, cycloheximide similarly renders TNF-α pro-apoptotic, suggesting that NF-κB activation controls the production of a protein(s) that protects eosinophils from the cytotoxic effects of TNF-α. Evidence is presented suggesting that TNF-α triggered apoptosis is more susceptible to NF-κB inhibition than constitutive apoptosis, leading to the possibility of the specific targeting of apoptosis in eosinophil sub-populations. Prior to morphological signs of apoptosis, TNF-α-induced IL-8 synthesis is abrogated by inhibition of NF-κB. We propose that NF-κB activation plays a critical role in controlling eosinophil responsiveness and apoptosis, and speculate that selective inhibitors of eosinophil NF-κB activation may ultimately provide alternative therapeutic agents for the treatment of eosinophilic diseases, including asthma and allergic rhinitis.

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1 Introduction

Eosinophilic granulocytes are normally involved in orchestrating the destruction and removal of invading parasites. However, if these cells are inappropriately recruited or activated at inflammatory sites they can liberate excessive amounts of toxic products (*e.g.* free O_2 radicals, major basic protein, etc.), which can cause damage to the surrounding tissue. Thus, eosinophil-mediated epithelial cell damage in the respiratory tract is likely to contribute to the pathogenesis and propagation of asthma and allergic rhinitis [1, 2]. Ligation of specific cell surface receptors and the consequent triggering of diverse signal transduction pathways control eosinophil functional responsiveness [3] and physiological programmed cell death (apoptosis) [4]. During apoptosis granulocytes undergo a series of dramatic morphological transformations (*e.g.* shrinkage and chromatin condensation) and surface molecule alterations (*e.g.* expression of phosphatidylserine) [5–8]. Apoptosis also causes a marked reduction in the cell's ability to respond to pro-inflammatory stimuli [5], and is critical for the efficient non-inflammatory [5, 6] removal of effete granulocytes from the inflammatory site by macrophages [7] and other cells including epithelial cells [9].

Eosinophils have the capacity to release inflammatory mediators derived from lipid metabolism and are capable of synthesizing pro-inflammatory cytokines [*e.g.* IL-8, TNF-α, granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-5, etc.] [3], many of which are regulated by the transcription factor nuclear factor-κB (NF-κB) [10–12]. Our recent demonstration that neutrophil apoptosis is critically regulated by the synthesis of NF-κB controlled survival protein(s) [13, 14] suggests that NF-κB may control gene transcription of proteins that influence granulocyte apoptosis as well the synthesis of pro-

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nflammatory agents. NF-κB1 (p50), NF-κB2 (p52), RelA p65), relB and c-rel are a family of proteins which bind to one another to form NF-κB dimers (often p50/p65), that eside predominantly in the cytoplasm. NF-κB is held in the cytoplasm in an inactive state by being bound to an nhibitory protein from the IκB family in which the 37-kDa κBα is the major form. When cells are appropriately stimulated, the IκBα subunit is rapidly phosphorylated and ubiquinated before finally being degraded by the 26S proteasome [10–12]. Translocation of NF-κB subunits to the nucleus, where they bind to regulatory DNA sequences in the upstream promoter region of target genes, controls rates of transcription [10, 11]. Inflammatory cells have the capacity to synthesize and respond to exogenous NF-κB regulated pro-inflammatory cytokines [15]. For example, TNF-α is capable of priming neutrophils, rendering them more susceptible to activation upon subsequent exposure to other neutrophil secretagogues [16, 17]. Furthermore, TNF-α can influence apoptosis in many cells including granulocytes [13, 18–20]; effects that may be controlled by activation of NF-κB [13, 18, 20]. For example, apoptosis is promoted when neutrophils are exposed to TNF-α for short periods of culture (*i.e.* 2–8 h) in a concentration-dependent manner. When apoptosis is assessed after 20 h of TNF-α exposure apoptosis is delayed [18]. Interestingly, TNF-α has the ability to induce apoptosis in human neutrophils via a mechanism where TNF receptor 2 (TNFR2) facilitates the dominant TNF receptor 1 (TNFR1) death effect. In human eosinophils TNF-α has been shown to exert an anti-apoptotic effect that signals partly via the p38 MAP kinase pathway [19], and more recently Temkin and Levi-Schaffer [20] have shown that TNF-α-mediated eosinophil survival occurs via ligation of both TNF receptor subtypes and through the generation of the eosinophil survival factor GM-CSF; an effect that seems to involve activation of NF-κB. Although we [13] and others [20, 21, 22] have shown that NF-κB is present in granulocytes, the role of NF-κB in the regulation of eosinophil-derived chemokine production and apoptosis remains poorly understood. We used two compounds that inhibit NF-κB activation; MG-132, a widely used proteasome inhibitor that prevents IκB degradation [23] and gliotoxin [13, 24]. Gliotoxin is an epipolythiodioxoperazine which exhibits immune suppressive activity both *in vitro* and *in vivo* [24, 25] and selectively inhibits NF-κB activation [24]. We demonstrate that both gliotoxin and MG-132 can un-mask the potential of TNF-α to induce eosinophil apoptosis. At the same concentration that affects apoptosis and inhibits TNF-α-induced IL-8 secretion, gliotoxin inhibits the translocation of p65 to the nucleus from the cytoplasm by inhibiting the degradation of IκBα. From our data we therefore propose that the NF-κB pathway plays a key role in regulation of eosinophil responsiveness and apoptosis; a system that may be modulated for therapeutic gain.

2 Results

2.1 NF-κB regulates eosinophil apoptosis

We have shown that NF-κB plays a central role in eosinophil apoptosis by demonstrating that blockade of NF-κB activation by gliotoxin un-masks the ability of TNF-α to dramatically induce eosinophil apoptosis. This is clearly depicted in Fig. 1A, where TNF-α (10 ng/ml) alone and gliotoxin (100 ng/ml) alone cultured with eosinophils for 4 h caused little morphological apoptosis; however, when eosinophils were cultured in the presence of TNF-α plus gliotoxin there was a dramatic induction of characteristic pyknotic nuclei typical of eosinophil apoptosis. Our preliminary data indicated that it requires a significant amount of time (>3 h) to observe significant apoptosis and that the 4-h time point is optimal for observing the synergistic effects of TNF-α and gliotoxin (data not shown). As an additional independent assessment of eosinophil apoptosis, we performed experiments in which surface changes associated with the apoptotic program were also assessed. For this we measured FITC-labeled annexin V binding in the presence of Ca^{2+} to phosphatidylserine molecules exposed on the outer membrane of apoptotic cells, where the annexin V 'low peak' represents non-apoptotic cells and the annexin V 'high peak' represents apoptotic cells. In agreement with morphological assessment of apoptosis, Fig. 1C shows that, although control and TNF-α-treated eosinophils at 4 h exhibit low rates of apoptosis, the small increase in annexin V^+ cells observed with gliotoxin alone is again dramatically augmented when the cells are cultured in the presence of both reagents together. Furthermore, the marked synergism that was displayed by gliotoxin and TNF-α is mediated by triggering of the caspase pathway since the broad-spectrum caspase inhibitor, zVAD-fmk (100 μM) completely blocked the pro-apoptotic effects induced by the combination of TNF-α plus gliotoxin (Fig. 2). When eosinophils were co-cultured with the protein synthesis inhibitor cycloheximide (5 μM), at a concentration and time point that had almost no direct effect on eosinophil apoptosis, a similar un-masking of the pro-apoptotic effect of TNF-α was observed; an effect that was also suppressed by zVAD-fmk (Fig. 2).

In addition, when eosinophils were cultured with the proteasome inhibitor MG-132 (20 μM) a marked induction of apoptosis was also observed, although the pro-apoptotic effect required longer incubation times than gliotoxin. For example, no significant induction of apoptosis was observed above constitutive levels when eosinophils were cultured for 8 h with MG-132 (data not shown). Even with incubation periods of 30 h, MG-132 induced little apoptosis; however, in the presence of TNF-α there was a dramatic synergistic induction of

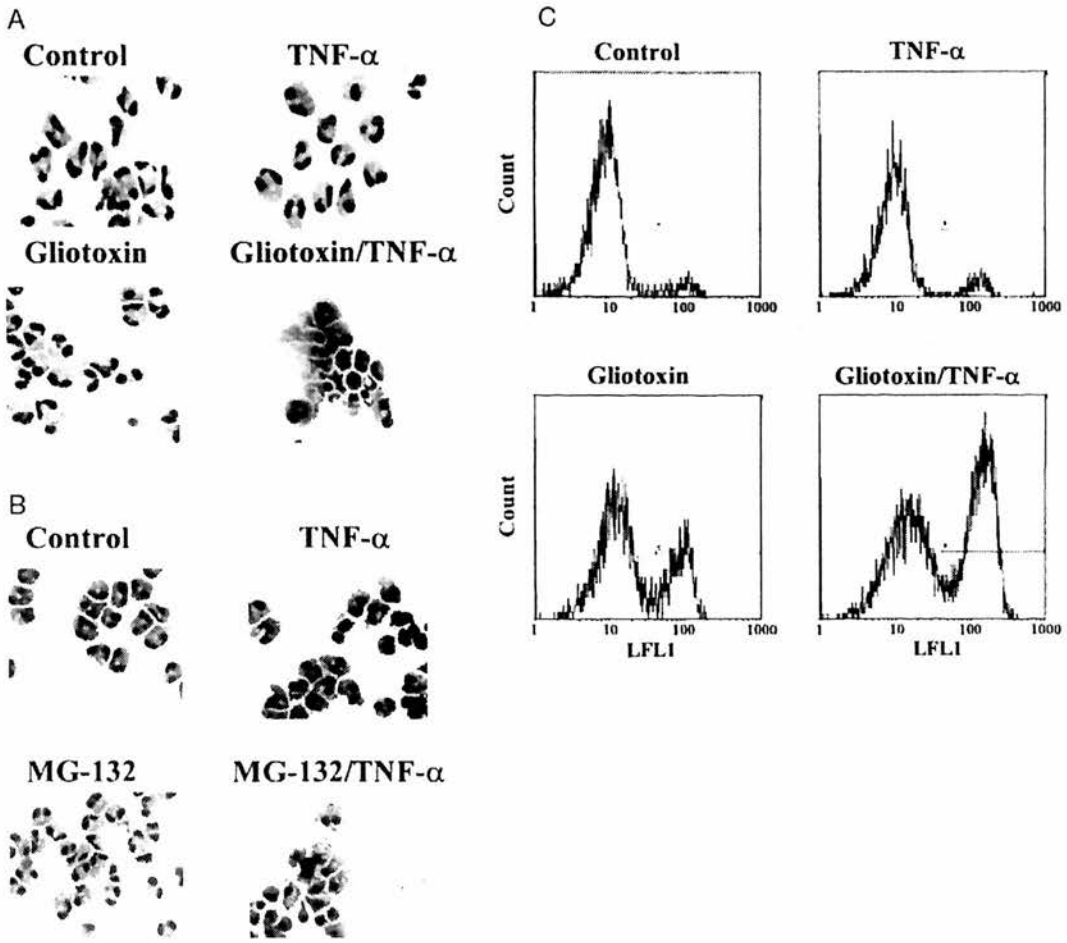

Fig. 1. The effect of gliotoxin, MG-132 and TNF-α on eosinophil apoptosis. (A) Cytocentrifuge preparations were made of human eosinophils incubated for 4 h at 37°C in medium alone (control), 10 ng/ml TNF-α, 100 ng/ml gliotoxin, and combination of these reagents. The rates of apoptosis in this experiment are control, 5%; TNF-α, 5%; gliotoxin, 26.5%; and gliotoxin/TNF-α, 47.5%. (B) Cytocentrifuge preparations of human eosinophils incubated for 30 h at 37°C in medium alone (control), 10 ng/ml TNF-α, MG-132 (20 μM), and combination of these reagents. The rates of apoptosis in this experiment are control, 14.6%; TNF-α, 23 3%; MG-132, 11.4%; and MG-132/TNF-α, 75.7%. (C) Aliquots (20 μl) were taken from human eosinophils that had been incubated for 4 h at 37°C in medium alone (control), 10 ng/ml TNF-α, 100 ng/ml gliotoxin, and a combination of these reagents and incubated with FITC-labeled recombinant human annexin V to determine the phosphatidylserine expression on the cell surface. Flow cytometric traces from a typical experiment are depicted with the percentage of cells in the annexin-V high population being 7.0% in control, 9.8% in TNF-α, 28.5% in gliotoxin and 50.8% in TNF-α plus gliotoxin-treated samples.

apoptosis (Fig. 1B). When apoptosis was assessed after 40-h treatment with MG-132 there was a significant induction of apoptosis (control, 24.8±8.7%; MG-132, 72.5±15.8% apoptosis; $p<0.05$, $n=4$). The pro-apoptotic effects of MG-132 *per se* and the enhanced apoptosis observed with a combination of MG-132 and TNF-α were also suppressed by zVAD-fmk (data not shown).

Taken together the above results support the view that TNF-α treatment results in the NF-κB controlled generation of a survival protein(s) that protects eosinophils from the TNF-α receptor- and caspase-dependent induction of apoptosis. To substantiate this hypothesis we performed a detailed direct analysis of NF-κB activation.

2.2 TNF-α induced cytoplasmic IκBα degradation and p65 disappearance in eosinophils

Upon appropriate cell stimulation, the inhibitory subunit of NF-κB, IκBα, is rapidly phosphorylated and undergoes proteolytic breakdown by the proteasome, thereby permitting NF-κB to translocate from the cytoplasm from

the nucleus [10]. Since the phosphorylation and subsequent degradation of IκBα is a prerequisite for NF-κB activation, the amount of IκBα in cytosolic extracts of eosinophils was examined by Western blotting to find evidence for the activation of the NF-κB pathway. In quiescent un-stimulated cells, IκBα appeared as a single band of 37 kDa, which disappeared following TNF-α stimulation, indicating degradation of IκBα (Fig. 3A) and translocation of NF-κB to the nucleus. Following TNF-α stimulation the amount of p65 in the cytoplasm was reduced (Fig. 3B), indicating that, following the degradation of IκBα, p65 is released from IκBα in the cytoplasm and translocated into the nucleus. As shown in Fig. 3A, gliotoxin had no direct effect on IκBα levels but inhibited TNF-α-induced disappearance of IκBα. The latter observation is consistent with gliotoxin-mediated inhibition of TNF-α-induced loss of cytoplasmic p65 (Fig. 3B). These results therefore suggest that TNF-α stimulation results in IκBα degradation and translocation of NF-κB from the cytoplasm to the nucleus.

2.3 Immunohistochemical analysis of NF-κB translocation in eosinophils

To provide compelling evidence for subcellular movement of NF-κB in eosinophils we performed quantitative immunohistochemical analysis of TNF-α-induced translocation of p65. Immunofluorescent confocal images of p65 (FITC: green) reveal translocation into the nucleus (propidium iodide: red) by TNF-α stimulation (shown as yellow as the result of overlapping green p65-FITC, and red propidium iodide) (Fig. 4) that is effectively prevented by gliotoxin. These results are consistent with the Western blot analysis shown in Fig. 3A and B, and therefore provide evidence that gliotoxin acts by inhibition of NF-κB activation. Quantification of translocation is depicted in Fig. 5A–C where the immunofluorescent images are analyzed on a Leica Q550IW image analyzer to detect p65 amount in the cytoplasm and nucleus and the results expressed as a ratio. Fig. 5A and B clearly show the inhibition of TNF-α-induced NF-κB translocation into the nucleus by gliotoxin and MG-132. In Fig. 5C the time course of translocation of p65 stimulated with TNF-α from a representative experiment is depicted. Maximum induction of TNF-α-stimulated p65 translocation in eosinophils isolated from this particular donor occurred after 60-min exposure to TNF-α as assessed by immunofluorescence. Following this time point, NF-κB levels in the nucleus rapidly decrease almost to basal levels, perhaps reflecting rapid re-synthesis of IκBα and consequent removal of NF-κB from the nucleus. Although different levels of basal and stimulated p65 distribution were observed between individual donors, substantial

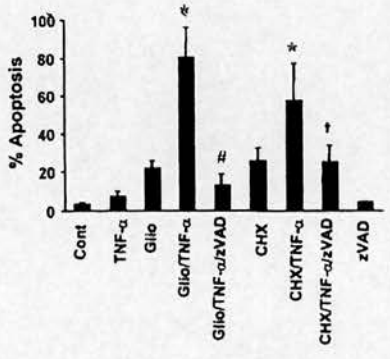

Fig. 2. Effects of the caspase inhibitor, zVAD-fmk, and the protein synthesis inhibitor, cycloheximide, on eosinophil apoptosis induced by gliotoxin, TNF-α or on a combination of these agents. Human eosinophils were treated with the indicated reagents for 4 h at 37°C before cytocentrifuge preparations were made and apoptosis assessed morphologically. Concentrations are: 10 ng/ml TNF-α, 100 ng/ml gliotoxin, 100 μM zVAD-fmk, and 5 μM cycloheximide (CHX). The data are expressed as the mean ± SEM of four separate experiments using eosinophils isolated from different donors. *Represents significance differences ($p<0.05$) from control, and # represents significance difference from Glio/TNF-α and † represents significance difference form CHX/TNF-α.

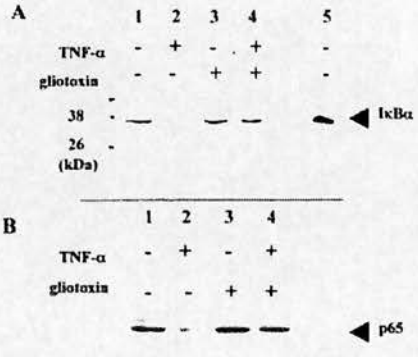

Fig. 3. Western blot analysis of cytoplasmic IκBα and p65. Eosinophils were stimulated with 10 ng/ml TNF-α, 100 ng/ml gliotoxin for 15 min in Iscove's DMEM at 37°C and cytoplasmic extract prepared and analyzed by Western blotting with anti-IκBα antibody, and anti-p65 antibody. (A) Cytoplasmic IκBα expression. Upon TNF-α stimulation the IκBα band disappears, indicative of proteosomal degradation; treatment of the cells with gliotoxin stabilizes IκBα in the cytoplasm both in the absence and presence of TNF-α. For a control Jurkat cell cytoplasmic extracts were included (lane 5). (B) The amount cytoplasmic p65. The loss of p65 expression in the cytoplasm with TNF-α stimulation correlated with IκBα loss and suggests translocation of p65 into the nucleus, a process blocked by gliotoxin. The blots are representative of at least three separate experiments.

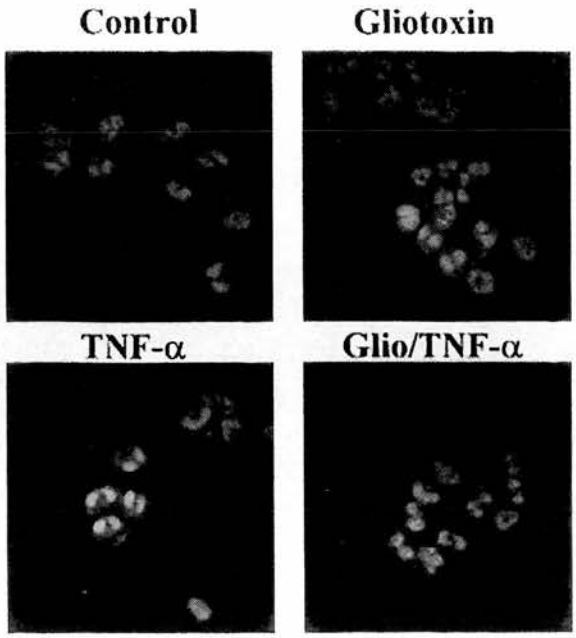

Fig. 4. Confocal microscope images of p65 expression in human eosinophils stimulated with TNF-α with and without gliotoxin. Eosinophils were stimulated for 45 min in Iscove's DMEM with 10 ng/ml TNF-α and 100 ng/ml gliotoxin. Anti-p65 antibody was FITC labeled (shown as green) and the nucleus was stained with propidium iodine (shown as red). Upon TNF-α stimulation p65 migrates into the nucleus, resulting in an enhanced yellow color. Gliotoxin, with or without TNF-α, dramatically inhibits p65 nuclear migration.

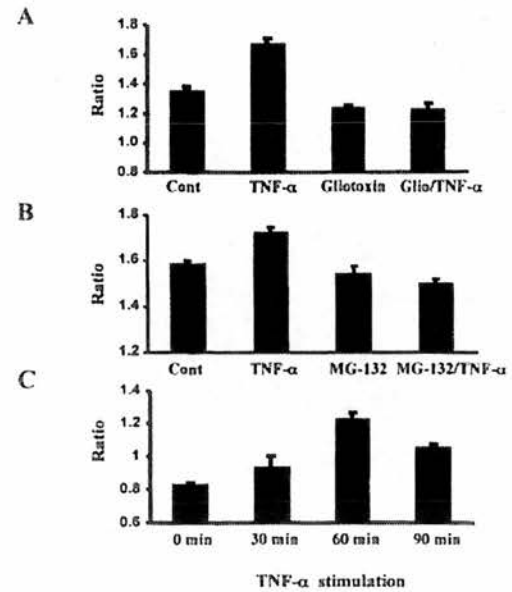

Fig. 5. Quantitative analysis of p65 translocation to the nucleus. Computer analysis of p65 expression in the nucleus was measured as the ratio of p65-FITC nuclear fluorescence versus cytoplasmic fluorescence in each cell. At least 300 cells were counted per condition and the data are represented as mean ± SEM determined for each experiment independently. Eosinophils were incubated in Iscove's DMEM at 37°C with the indicated stimuli (A and B) for 45 min and with TNF-α for the indicated times (C). (A) The effect of 100 ng/ml gliotoxin on activation by 10 ng/ml TNF-α. (B) The effect of 20 μM MG-132 on p65 translocation into the nucleus. (C) Time course of the effect of 10 ng/ml TNF-α. The data are representative of one experiment of at least three similar experiments.

NF-κB activation by TNF-α stimulation was observed (data not shown).

2.4 NF-κB activation mediates IL-8 production by human eosinophils

Having clearly shown the presence and activation of the NF-κB pathway in eosinophils and demonstrated that this pathway regulates the eosinophil apoptotic program in response to TNF-α, we next sought to investigate its role in regulating eosinophil responsiveness. It is established in many cell types [11, 26] that IL-8 production is tightly controlled by NF-κB, and eosinophils have the capacity to synthesize this important pro-inflammatory cytokine [3, 21]. TNF-α is a powerful stimulator of IL-8 production in eosinophils (Fig. 6) even at the early time point of 90 min, a time point chosen since there is no gliotoxin-induced apoptosis that could influence IL-8 production (data not shown). The production of IL-8 was dramatically suppressed by gliotoxin at a concentration that effectively inhibits NF-κB activation at this time point where there is no significant apoptosis.

3 Discussion

We have clearly demonstrated that human eosinophils possesses a fully functional NF-κB signaling system that plays an important role in regulating cellular responsiveness and apoptosis. Our data show that the eosinophil contains p65 and that NF-κB is presumably maintained in the cytoplasm by the inhibitor protein IκBα. Upon exposure to the pro-inflammatory agent TNF-α, IκBα is rapidly lost from the cytoplasm by a process that involves phosphorylation and ubiquitination, followed by subsequent degradation by the proteasome. In eosinophils, Western blot analysis and immunofluorescence data demonstrate that p65 translocates from the cytoplasm to the nucleus in response to TNF-α. One likely partner for p65 in eosinophils is p50, as identified by Western blot analysis ([21] and data not shown). Gliotoxin and MG-132 effectively prevents p65 translocation to the nucleus, which we suggest is important for the

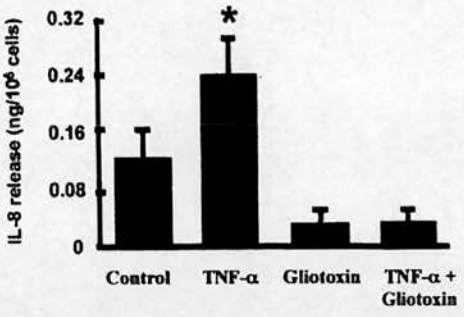

Fig. 6. Effect of gliotoxin on TNF-α-induced IL-8 release from human eosinophils. Eosinophils were stimulated with 10 ng/ml TNF-α for 90 min in the absence and presence of 100 ng/ml gliotoxin and the IL-8 concentrations in the cell supernatants determined by specific ELISA. The data are expressed as the mean ± SEM of four separate experiments using eosinophils isolated from different donors. Significant increase ($p<0.05$) from the control is indicated by *.

direct effect of these compounds on apoptosis. TNF-α is a powerful activator of NF-κB and gliotoxin and MG-132 inhibit NF-κB activation upon TNF-α stimulation resulting in a dramatic synergistic induction of apoptosis. Interestingly, the following observations suggest that TNF-α-triggered apoptosis is more susceptible to NF-κB inhibition than constitutive apoptosis; firstly, higher concentrations of gliotoxin are required to directly induce apoptosis than required to render the cells responsive to the pro-apoptotic effects of TNF-α [13] and secondly MG-132 augments TNF-α-induced apoptosis at earlier time points than are required to induce apoptosis directly. These findings clearly require further investigation but provide the intriguing possibility of selectively targeting the removal of potentially histotoxic eosinophils from inflammatory sites by manipulating apoptosis of such eosinophil sub-populations without influencing constitutive tissue eosinophils.

The NF-κB complex binds to DNA regulatory sites on the upstream (5') promotor sequences of target genes that control the rate of gene transcription for many pro-inflammatory mediators, some of which can influence granulocyte function and apoptosis (*e.g.* TNF-α and IL-8). Eosinophils exposed to TNF-α (Fig. 6) and lipopolysaccharide [27] are capable of releasing IL-8. IL-8 itself can cause recruitment of inflammatory cells into sites of inflammation and can also prime and/or activate granulocytes [28, 29]. Thus activation of the NF-κB pathway can augment the inflammatory response by generating further pro-inflammatory mediators. In our studies, synthesis of IL-8 is inhibited by relatively low concentrations of gliotoxin even at a time point (90 min) at which there is no significant induction of apoptosis, consistent with the observation that TNF-α activates NF-κB to induce IL-8 production in human eosinophils (Fig. 6; [21]). Although we have shown previously that gliotoxin, but not its structurally similar analog, methylthiogliotoxin, can directly induce neutrophil apoptosis and enhance apoptosis in the presence of TNF-α [13] by selectively inhibiting NF-κB activation, the underlying mechanisms were not identified. We show here that gliotoxin not only prevents translocation of p65 from the cytoplasm to the nucleus but also prevents IκBα degradation. Interestingly, MG-132, like gliotoxin, can also induce eosinophil apoptosis directly and also renders eosinophils responsive to the pro-apoptotic effects of TNF-α. It is noteworthy that MG-132 required a longer incubation period in comparison to gliotoxin to un-mask TNF-α-induced apoptosis. The precise reason for this is unknown but may reflect a difference in the mechanism of action or in the rates of cellular incorporation between the two inhibitors of NF-κB activation. We do, however, propose that NF-κB activation is a critical regulator of granulocyte apoptotic programs. Studies using cycloheximide suggests that granulocytes synthesize a survival protein(s) that inhibits apoptosis [30]. Our results showing that cycloheximide dramatically un-masks the pro-apoptotic effect of TNF-α in eosinophils are entirely consistent with our hypothesis that NF-κB activation leads to the synthesis of a survival protein that limits the ability of the cell to undergo apoptosis. Thus, TNF-α triggers a number of signaling pathways following ligation of TNF-α receptors. We have previously shown that neutrophils undergo an early induction of apoptosis (2–8 h) and a later (>18 h) inhibition of apoptosis when cultured *in vitro* in the presence of TNF-α [18]. The pro-apoptotic effect of TNF-α is thought to be mediated by ligation of the TNFR1 containing a death domain that interacts with the TNF-receptor-associated death domain protein (TRADD) allowing recruitment of Fas-associated death domain (FADD), leading to activation of the caspase enzyme pathway. This highly regulated sequence of events enables apoptosis to be triggered. TNFR 2, often in co-operation with TNFR1, results in the activation of many kinases including the p38-Jun N-terminal kinase (JNK) pathway and IKK [31]. Prevention of the survival pathway by blocking the generation of NF-κB-mediated protein synthesis allows the death pathway to predominate and a massive induction of apoptosis is observed. It remains possible that blockade of NF-κB will prevent the synthesis of powerful NF-κB-regulated survival cytokines such as GM-CSF, IL-5 and IL-3. Indeed, it has very recently been published that TNF-α via ligation of both TNFRI and TNFR2 subtypes mediated eosinophil survival (as assessed by Trypan blue exclusion), and that this occurred through regulation of GM-CSF but not IL-3 and IL-5 production [20]. Furthermore, it was shown that GM-CSF is indeed synthesized when eosinophils are challenged with TNF-α and that TNF-α enhanced eosin-

ophil survival is significantly inhibited by the proteasome inhibitor MG-132 [20]. It also remains likely that eosinophils like other cells can generate protein(s) that directly influence the apoptotic program. However, the precise identity of the potential survival protein(s) is unknown (see [14] for a review); however, several likely candidate proteins have been implicated including members of the oncogene Bcl2 family (*e.g.* A1 [32, 33] and Mcl-1 [34]) and others such as c-Myc [35], A20 [36], cIAP [37, 38], and IEX-1L [39].

Although it is abundantly clear that NF-κB activation can play an important role in regulating the production of cytokines and adhesion molecules that are vital for orchestrating the inflammatory response, there is relatively little direct *in vivo* evidence indicating that this transcription factor can influence eosinophilic inflammation. Compelling evidence that NF-κB plays an essential role in the induction of eosinophilia in allergic airway inflammation, was obtained using mice deficient in p50, when compared with wild-type mice [40]. In addition to being incapable of mounting eosinophilic airway inflammation, p50–/– mice had a dramatically reduced capacity for the production NF-κB regulated inflammatory mediators such as the T helper 2 cytokine IL-5 and the chemokines eotaxin and macrophage inflammatory protein (MIP)-1α and MIP-1β. We believe that NF-κB plays a prominent role in controlling eosinophil responsiveness and longevity, and therefore could have far reaching implications for understanding not only the generation and propagation, but also the resolution, of eosinophilic inflammation. Further investigations may lead to the development of novel compounds that interfere with the NF-κB signaling pathway which may be applied to clinically relevant eosinophilic diseases such as asthma and allergic rhinitis.

4 Materials and methods

4.1 Eosinophil isolation and culture

Granulocytes were isolated from the peripheral blood of normal or mildly atopic donors by dextran sedimentation followed by centrifugation through discontinuous Percoll (Amersham/Pharmacia, Little Chalfont, GB) gradients [13, 41]. Eosinophils were separated from contaminating neutrophils by negative selection using an immunomagnetic separation step with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450, Dynal, Merseyside, GB) coated with the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York) as described [42]. Eosinophils were routinely >98% pure and >99% viable. After purification, eosinophils were washed twice in PBS without Ca^{2+} and Mg^{2+} and resuspended as indicated below. Eosinophils in Iscove's Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley, GB) supplemented with 10% autologous serum were cultured in Falcon flat-bottom flexible 96-well plates (Becton Dickinson, Oxford, GB) at 37°C in a 5% CO_2 atmosphere at 2×10^6/ml. Cells were cultured in the absence or presence of test agents as described in the figure legends.

4.2 Assessment of eosinophil apoptosis

4.2.1 Morphology

Cells (120 μl of 2×10^6/ml) were cyto-centrifuged, fixed in methanol, stained with Diff-Quik™ (Gamidor Ltd., Abingdon, GB) and counted using oil immersion microscopy (×100 objective) to determine the proportion of cells with distinctive apoptotic morphology [4, 7, 13]. At least 500 cells were counted per slide. All experiments were performed at least three times and each treatment done in duplicate and the results are expressed as the mean % apoptosis ± SEM.

4.2.2 Annexin V binding

A separate and independent assessment of apoptosis was performed by flow cytometry using FITC-labeled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V (Bender MedSystems, Vienna, Austria) was diluted 1:200 with binding buffer and then added (20 μl) to 120 μl of the recovered cell samples. Following a 10-min incubation at 4°C, these samples were fixed by the addition of 100 μl of 3% paraformaldehyde in PBS before analysis using an EPICS Profile II (Coulter Electronics, Luton, GB) [13].

4.3 Preparation of cell cytoplasmic extracts

Following incubation at 37°C with the indicated reagents (see figure legends for concentrations and times) eosinophils were washed with ice-cold PBS and the cells lysed at 4°C for 15 min with Nonidet P-40 lysis buffer as described [43] (50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 mM β-glyceropyrophosphate, 0.5 % (v/v) Nonidet P-40, 2 mM EDTA, 16.8 mM Na_2HPO_4, 3.2 mM NaH_2PO_4) containing complete protease inhibitor cocktail EDTA free (Roche Diagnostics, Mannheim, Germany). The cell fragments were then centrifuged (13,000×g, 10 min, 4°C) and the supernatants containing the cytoplasmic extracts collected.

4.4 Western blot analysis

The cell extracts were diluted with 3x sample buffer [5.7% (wt/v) SDS, 14% mercaptoethanol, 150 mM Tris-HCl, pH 6.7; 30% (v/v) glycerol] and heated at 95°C for 3 min before running on 10% (wt/v) SDS-polyacrylamide gel. Electophoretically separated proteins were transferred on PVDF microporous membranes (Millipore Corporation). Non-specific

binding was blocked with PBS containing 5% non-fat dry milk and 0.1% Tween 20 (Sigma Co., Poole, GB) and probed with the indicated antibodies (anti-IκBα from New England Biolab Ltd., Hertfordshire, GB; anti-p65 and -p50 antibodies from Santa Cruz Biotechnology, CA). As a positive control 10 μg of protein extracted from un-treated Jurkat cell [maintained in RPMI 1640 (Life Technologies), 10% FCS (Life Technologies)] extract was also run on the gels. Immunodetected proteins were visualized by ECL™ detection reagents (Amersham/Pharmacia).

4.5 Immunohistochemistry

Eosinophils (2.5×10^6/ml) were incubated in Iscove's DMEM containing 5% FCS for 2 h at 37°C before treatment with the indicated agents and time (see figure legends for details). The cells were cyto-centrifuged, air dried for 10 min and then fixed with 4% (w/v) p-formaldehyde/PBS for 10 min and washed three times with PBS. The cells were permeabilized and nonspecific binding was blocked in buffer containing 0.2% (w/v) Triton X-100 protein block serum-free buffer (DAKO Corp., Glostrup, Denmark) at room temperature for 30 min. The cells were incubated with rabbit polyclonal p65 antibody (Santa Cruz Biotechnology; 1:100 dilution) in antibody diluent (DAKO) with 0.2% Triton X-100 for 1 h. The cells were washed three times in antibody diluent with 0.2% Triton X-100 and then incubated (1:100 dilution) with Alexa™ 488 (goat anti-rabbit) IgG antibody (Molecular Probes, Eugene, OR) for 1 h in antibody diluent with 0.2% Triton X-100 then washed three times in the same buffer. The nucleus was stained with 0.1% (v/v) propidium iodide (Sigma) with 2% (w/v) RNase in PBS at 37°C for 30 min followed by washing (×3) with PBS before applying a glass coverslip.

4.6 Confocal microscopy and image analysis

Images are from single confocal sections, 0.3 μm thick, taken through the center of the nucleus, on a Leica TSC 4D confocal microscope using a 63× objective lens. NF-κB nuclear translocation was determined with a Leica DMRB microscope using a 40× NA 0.7 objective lens and Kappa integrating monochrome CCD camera. Image analysis was integrated with image capture as described before [44]. The image analyzer captures an image of the green NF-κB fluorescence and using an automated macro-program developed for this application on a Leica Q550IW image analyzer and uses the binary images of the propidium iodide staining as a mask to measure only the NF-κB fluorescence of the nuclei. The image analyzer then detects the green fluorescence (488 nm excitation, 530 nm emission) and subtracts the binary image of the red fluorescence (570 nm excitation, 620 nm emission) to give a binary mask for the cytoplasm. At least 300 cells were counted per condition and the data accumulated. Means ± SEM are determined for each experiment independently.

4.7 IL-8 measurement

The ELISA was performed using IL-8 recombinant protein as a standard and IgG anti-human IL-8 according to the manufacture's instructions (R&D systems, Abingdon, GB).

4.8 Materials

Further specific materials were obtained as follows: recombinant human TNF-α (R&D Systems), MG-132 (N-cbz-Leu-Leu-leucinal) (Biomol, Affinity Research Products, Mamhead, GB) and zVAD-fmk (benzylocarbonyl-Val-Ala-Asp-fluoromethylketone) (Bachem Ltd., Saffron Walden, GB).

4.9 Statistical analysis

The results are expressed as the mean ± SEM of the number (n) of independent experiments each using cells isolated from different donors with each treatment performed in duplicate or triplicate. Statistical analysis was performed by ANOVA with comparisons between groups made using the Newmans-Kuels procedure. Where appropriate, analysis was performed using the paired Student's t-test. Differences were considered significant when $p<0.05$, and are represented by the indicated symbol.

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NF-κB Activation Is a Critical Regulator of Human Granulocyte Apoptosis *in Vitro**

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During beneficial inflammation, potentially tissue-damaging granulocytes undergo apoptosis before being cleared by phagocytes in a non-phlogistic manner. Here we show that the rate of constitutive apoptosis in human neutrophils and eosinophils is greatly accelerated in both a rapid and concentration-dependent manner by the fungal metabolite gliotoxin, but not by its inactive analog methylthiogliotoxin. This induction of apoptosis was abolished by the caspase inhibitor zVAD-fmk, correlated with the inhibition of nuclear factor-kappa B (NF-κB), and was mimicked by a cell permeable inhibitory peptide of NF-κB, SN-50; other NF-κB inhibitors, curcumin and pyrrolidine dithiocarbamate; and the proteasome inhibitor, MG-132. Gliotoxin also augmented dramatically the early (2–6 h) pro-apoptotic effects of tumor necrosis factor-α (TNF-α) in neutrophils and unmasked the ability of TNF-α to induce eosinophil apoptosis. In neutrophils, TNF-α caused a gliotoxin-inhibitable activation of an inducible form of NF-κB, a response that may underlie the ability of TNF-α to delay apoptosis at later times (12–24 h) and limit its early killing effect. Furthermore, cycloheximide displayed a similar capacity to enhance TNF-α induced neutrophil apoptosis even at time points when cycloheximide alone had no pro-apoptotic effect, suggesting that NF-κB may regulate the production of protein(s) which protect neutrophils from the cytotoxic effects of TNF-α. These data shed light on the biochemical and molecular mechanisms regulating human granulocyte apoptosis and, in particular, indicate that the transcription factor NF-κB plays a crucial role in regulating the physiological cell death pathway in granulocytes.

Neutrophilic and eosinophilic granulocytes originate from a common myeloid precursor; neutrophils are particularly active in the defense against invading micro-organisms whereas eosinophils serve in anti-parasitic defenses and play a role in allergic inflammation. The normally beneficial acute inflammatory response can become dysregulated and result in chronic inflammatory conditions where tissue damage arises in part due to the inappropriate liberation of inflammatory cell-derived histotoxic products. We have previously described a granulocyte clearance mechanism likely to be important in the normal control and resolution processes of inflammation whereby granulocytes must first undergo apoptosis (programmed cell death) before being phagocytosed and cleared by macrophages *in situ* (1, 2). Apoptosis also causes functional down-regulation of granulocytes and the retention of proteolytic granule contents to further limit the potential for granulocyte-mediated tissue damage (3, 4). While little is known about the physiological mechanisms involved in controlling granulocyte apoptosis, many *in vitro* studies have now shown that an array of pro-inflammatory cytokines and inflammatory mediators known to be present at inflamed sites inhibit the process of apoptosis in granulocytes (2–9). This has led to the suggestion that such agents act both to attract and activate inflammatory cells and also delay their removal. A notable exception to this rule, however, is TNF-α,[1] which, at early time points in neutrophil culture, causes acceleration of the constitutive rate of apoptosis (10).

Many inflammatory mediators regulate gene expression in target cells by influencing the activities of transcription factors such as nuclear factor-κB (NF-κB). NF-κB is composed of homo- or heterodimers of the Rel family proteins (p50/NFκB1, p52/NFκB2, p65/RelA, and cRel) which are sequestered in the cytoplasm by physical association with inhibitor proteins referred to as IκB (11). Upon activation, the IκB subunit is rapidly phosphorylated leading to its proteolytic breakdown permitting NF-κB to translocate to the nucleus (12, 13) where it regulates the activity of many genes involved in the inflammatory response, including those for pro-inflammatory cytokines. In a number of cell systems TNF-α has been shown to induce rapid activation of NF-κB, a response known to mediate a number of TNF-α induced cellular responses (reviewed in Refs. 14–16). However, whether the activation of NF-κB is involved in either the pro- or anti-apoptotic effects of TNF-α in granulocytes is currently unknown.

Whereas the inhibition of NF-κB has been shown to induce apoptosis in murine B cells (17), a completely opposite effect has been observed in other cell types where apoptosis is associated with activation of NF-κB (18). In addition, several groups have reported that inactivation of NF-κB increases the cytotoxic effects of TNF-α (19–21). In granulocytes, it remains uncertain whether NF-κB can be activated by inflammatory mediators, or is indeed present, in human neutrophils. For

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; DMEM, Dulbecco's modified Eagle's medium.

example, while MacDonald *et al.*, (22) reported that lipopolysaccharide (LPS), TNF-α, and the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine all cause a marked activation of NF-κB, Browning *et al.*, (23) found no such activity in these cells despite obvious NF-κB activation in peripheral blood mononuclear cells.

Gliotoxin, a member of the epipolythiodioxoperazine family of compounds (24), exhibits immunosuppressive activity both *in vivo* and *in vitro*. For example, gliotoxin has been shown to inhibit mitogen-induced proliferation of both T and B cells, induce macrophage and osteoclast apoptosis *in vitro* (25, 26), and cause thymocyte and spleen cell apoptosis *in vivo* (27). However, the biochemical and molecular mechanisms underlying these effects remain uncertain. Gliotoxin has, however, recently been shown to be a potent and specific inhibitor of NF-κB (28). We therefore used gliotoxin as a pharmacological tool to investigate the involvement of NF-κB in the regulation of granulocyte apoptosis. We demonstrate that gliotoxin causes a rapid and major induction of apoptosis in human peripheral blood granulocytes *in vitro* and up-regulates TNF-α-induced apoptosis in both neutrophils and eosinophils. In addition, we present evidence that these effects occur via a specific, non-toxic and caspase-controlled mechanism that is mediated by the ability of gliotoxin to inhibit an inducible form of NF-κB. The ability of other NF-κB inhibitors to cause a similar induction of apoptosis provides further evidence supporting the involvement of NF-κB in granulocyte apoptosis. Interestingly, the pro-apoptotic effect of TNF-α is enhanced by protein synthesis blockade suggesting that NF-κB activation results in the generation of an unidentified survival protein. These data therefore strongly suggest that NF-κB plays a key role in regulating both constitutive and TNF-α stimulated human granulocyte apoptosis.

EXPERIMENTAL PROCEDURES

Neutrophil and Eosinophil Isolation and Culture

Neutrophils and eosinophils were isolated from the peripheral blood of normal donors by dextran sedimentation followed by centrifugation through discontinuous plasma-Percoll gradients (2, 29). Only neutrophil preparations with a neutrophil purity of >98% were used. Eosinophils were separated from contaminating neutrophils using an immunomagnetic separation step with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450, Dynal, Merseyside, United Kingdom) coated with the murine anti-neutrophil antibody 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York). Cells were mixed with washed 3G8-coated Dynabeads at a bead:neutrophil ratio of 3:1 on a rotary mixer at 4 °C for 20 min, and the beads removed magnetically by two 3-min stationary magnetic contacts (Dynal Magnetic Particle Concentrator, MPC-1) to yield an a eosinophil population of >98% purity. After purification, cells were washed twice in phosphate-buffered saline without calcium and magnesium and once in phosphate-buffered saline before resuspending in Iscove's DMEM (Life Technologies, Paisley, UK) with 10% autologous serum. Both cell types were cultured in flat-bottomed Falcon flexible wells (Becton Dickinson, Oxford, UK) at 37 °C in a 5% CO_2 atmosphere; neutrophils at a concentration of 5×10^6/ml and eosinophils at 2×10^6/ml. Cells were cultured in the absence or presence of test agents as described in the figure legends. All experiments were performed at least 3 times and each treatment done in triplicate.

Assessment of Granulocyte Apoptosis

Morphology—Cells were cyto-centrifuged, fixed in methanol, stained with Diff-Quik™, and counted using oil immersion microscopy (×100 objective) to determine the proportion of cells with highly distinctive apoptotic morphology (5, 7, 10). At least 500 cells were counted per slide with the observer blinded to the experiment conditions. The results were expressed as the mean % apoptosis ± S.E.

Annexin V Binding—A separate and independent assessment of apoptosis was performed by flow cytometry using fluorescein isothiocyanate-labeled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells. Stock annexin V (Bender MedSystems, Vienna, Austria) was diluted 1:200 with binding buffer and then added (25 μl) to 75 μl of the recovered cell samples. Following a 10-min incubation at 4 °C, these samples were fixed by the addition of 100 μl of 3% paraformaldehyde in phosphate-buffered saline before analysis using an EPICS Profile II (Coulter Electronics, Luton, UK).

DNA Fragmentation Assay—DNA was extracted as described previously (10). Briefly, 2×10^6 neutrophils were taken after the indicated treatment and lysed in 500 μl of lysis buffer (6 M guanidine hydrochloride, 50 mM Tris-HCl, pH 8.0, and 0.1% *N*-lauroyl sarcosine) at 4 °C and the nucleic acids extracted by the addition of an equal volume of 10 mM Tris-HCl, pH 8.0-saturated phenol:chloroform mixture (50:50, v/v). The resulting emulsion was centrifuged at $12,000 \times g_{av}$ for 10 min at room temperature and the aqueous phase removed and precipitated with 0.6 volumes of isopropyl alcohol at room temperature. The precipitated nucleic acids were then pelleted by centrifugation at $10,000 \times g_{av}$ for 5 min and re-dissolved in 50 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 50 μg/ml RNase A. The fragmented DNA was separated by agarose gel electrophoresis on a 1.4% (w/v) agarose (Flowgen, UK) 0.5 × TBE (10 mM Trizma (Tris base), 10 mM boric acid, and 1 mM EDTA, pH 8.3) gel. The gel was run for 2 h at 75 V and stained using ethidium bromide (0.5 μg/ml). The UV transilluminated image was printed by digital thermal printing using a GS7600 gel documentation system (UVP Products, UK).

Assessment of Cell Membrane Integrity

Since apoptotic neutrophils and eosinophils maintain the integrity of their plasma membrane, assessment of granulocyte necrosis can be determined by the ability of cells to exclude the vital dye trypan blue and also by flow cytometry using propidium iodide staining. Samples (150 μl of cells at 2×10^6/ml) were centrifuged and resuspended in 150 μl of propidium iodide solution (33 μg/ml propidium iodide in phosphate-buffered saline containing 1.67 mg/ml RNase). The profiles of heat-treated (necrotic cells) from the same samples were used as controls.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were carried out as described by the manufacturer (Promega Corp, Southampton, UK). Nuclear extracts were prepared from 5×10^6 cells using a modification of the method of Dignam *et al.* (30). Briefly, pelleted cells were resuspended in 200 μl of hypotonic buffer (buffer A: 10 mM Tris-HCl, pH 7.8, 1.5 mM EDTA, 10 mM KCl, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, leupeptin, and pepstatin A, 1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM benzamidine, and 2 mM levamisole) and placed on ice for 10 min. Following the addition of 0.1 volumes of 10% Nonidet P-40 (W/v) the cells were vortexed briefly and centrifuged at $12,000 \times g_{av}$ for 2 min at 4 °C. The supernatant was discarded and the pellet washed in 100 μl of buffer A minus Nonidet P-40 and re-centrifuged. The pelleted nuclei were then resuspended in 50 μl of hypertonic buffer (buffer B: 20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 50 mM KCl, 1.5 mM EDTA, 5 mM dithiothreitol, 1 μg/ml aprotinin, leupeptin, and pepstatin A, 1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM sodium orthovanadate, 0.5 mM benzamidine, and 2 mM levamisole) and stored at −80 °C until use.

Nuclear extracts (approximately 2 μg of protein, 7×10^5 cell equivalent, in 7 μl) were incubated in binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, with 50 μg/ml poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech, UK) with 17 fmol of γ^{-32}P-labeled double stranded oligonucleotide containing the decameric κB-binding site (3000 Ci/mmol; Promega Corp., Southampton, UK) at 22 °C for 20 min prior to addition of 5 μl of loading buffer (0.01% (w/v) bromphenol blue, 20% (w/v) Ficoll 400™, and 1 mM EDTA). Samples were loaded onto an 8% (w/v) native acrylamide gel (Protean IIxi, Bio-Rad, Hemel Hempstead, UK) in 0.5 × TBE buffer and run at 250 V for 2 h. The gel was then dried onto 3MM paper (Whatman UK, Maidstone, UK) and BioMax MS-1 x-ray film (Kodak, Anachem, Luton, UK) was exposed to the gel. Processed films were analyzed using the GrabIt and GelPlate software (UVP, Orme Technologies, UK) and the data expressed as a percent of the control value for each experiment.

Materials

Further specific materials were obtained as follows: curcumin, gliotoxin, LPS (*Escherichia coli* 0127:B8), methylthiogliotoxin, and PDTC (Sigma Co., Poole, UK); recombinant human TNF-α (R & D Systems, Abingdon, Oxon, UK); zVAD-fmk (Bachem (UK) Ltd., Saffron Walden, UK). The proteasome inhibitor MG-132 (N-cbz-Leu-Leu-leucinal), and the NF-κB inhibitory peptides SN50 and SN50M (Biomol, Affinity Re-

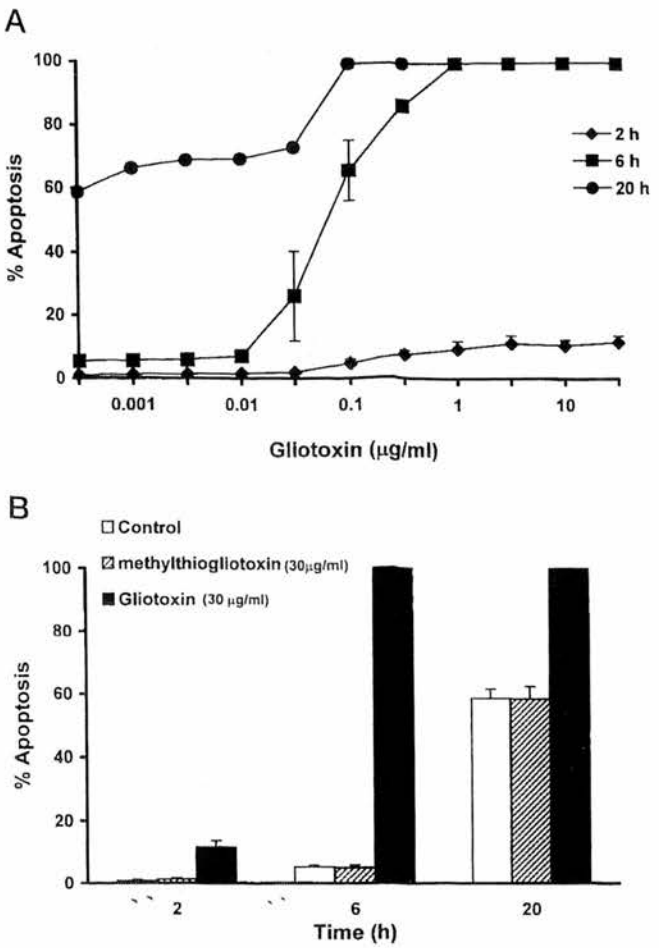


FIG. 1. **Time course and concentration-response curve for the effect of gliotoxin on human neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with the indicated concentrations of gliotoxin. At the time periods indicated, the cells were resuspended and cytocentrifuge preparations made. These were fixed and stained, and apoptosis was assessed morphologically. *A*, represents the effect of gliotoxin (0.001–30 μg/ml) on neutrophil apoptosis after 2, 6, or 20 h of culture. *B*, represents the effect of methylthiogliotoxin (30 μg/ml) or gliotoxin (30 μg/ml) on neutrophil apoptosis after 2, 6, or 20 h culture. All values represent mean ± S.E. of $n = 3$ experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.

search Products, Mamhead, UK). All other reagents were obtained from Sigma, UK, and were of the highest purity.

Statistical Analysis

The results are expressed as mean ± S.E. of the number (n) of independent experiments each using cells from separate donors with each treatment performed in triplicate. Statistical analysis was performed by ANOVA with comparisons between groups made using the Newman-Kuels procedure. Differences were considered significant when $p < 0.05$.

RESULTS

Effect of Gliotoxin on Neutrophil Apoptosis—As shown in Fig. 1 gliotoxin caused a rapid and profound induction of neutrophil apoptosis *in vitro* which was both concentration (*e.g.* at 6 h $EC_{50} = 76.1 \pm 22.1$ ng/ml, Fig. 1*A*), and time-dependent (Fig. 1, *A* and *B*). Hence using a maximally effective gliotoxin concentration of 1 μg/ml, apoptosis was readily apparent within 2 h and reached 100% by 6 h. At 20 h, when the rate of constitutive neutrophil apoptosis was 58.7 ± 2.9%, gliotoxin caused 100% apoptosis at all concentrations greater than 0.1 μg/ml. The inactive analogue of gliotoxin, methylthiogliotoxin, did not affect the constitutive rate of neutrophil apoptosis at any of the time points studied (Fig. 1*B*). Neither gliotoxin nor its inactive analogue, methylthiogliotoxin, caused cell necrosis since less than 1% of the cells were permeable to the vital dye trypan blue.

Gliotoxin Acts Synergistically with TNF-α to Stimulate Neutrophil Apoptosis—In contrast to many other hematopoetic cells, human neutrophils appear highly resistant to the induction of apoptosis induced by certain agents, for example, incubation with Ca^{2+} ionophores (31, 32), cAMP elevating agents (33), corticosteroids (9), and LPS (7) causes inhibition of apoptosis as does hypoxia (34). Furthermore, while TNF-α and Fas-L can induce neutrophil apoptosis, this effect is modest and transient, and in the case of TNF-α abolished if the cells are initially primed with platelet-activating factor or LPS (10, 35). We therefore sought to determine the effect of gliotoxin on TNF-α-induced apoptosis in neutrophils. These experiments were performed deliberately at a very early time point (2 h) when the independent pro-apoptotic effects of even a maximally effective concentration of TNF-α (10 ng/ml) (10) and gliotoxin (1 μg/ml; Fig. 1*A*) are only just apparent. As shown in Fig. 2*A*, a major synergy was observed between these agents for the induction of apoptosis which was apparent even at gliotoxin concentrations as low as 3 ng/ml. Hence, a concentration of 0.1 μg/ml gliotoxin in combination with TNF-α (10 ng/ml) caused almost 100% apoptosis at 2 h. With gliotoxin alone, only 6% apoptosis was noted at 2 h, with just over 65% at 6 h (Fig. 1*A*). Again, methylthiogliotoxin had no effect on constitutive apoptosis or TNF-α-induced apoptosis at 2 h (Fig. 2*B*). The level of necrosis in cells from each treatment was assessed by trypan blue exclusion; all values were <1% (data not shown).

The genuine nature of both the intrinsic pro-apoptotic effect of gliotoxin and the dramatic synergy with TNF-α was assessed by comparing the quantitative morphological effects of these agents with their effects on annexin V binding and DNA fragmentation. The changes from normal cell morphology to apoptotic morphology are clearly seen in Fig. 3*A*; where non-apoptotic neutrophils contain a multilobed nucleus and the apoptotic cells have a shrunken appearance with pyknotic nuclei. These data can be compared with Fig. 3*B*, where the annexin V "low peak" represents non-apoptotic cells and the annexin V "high peak" represents apoptotic cells since the fluorescein isothiocyanate-labeled annexin V binds in the presence of Ca^{2+} to phosphatidylserine exposed on the outer membrane of apoptotic cells. Although control cells at 2 h exhibit low rates of apoptosis, the small increase in annexin V positive cells observed with TNF-α and gliotoxin alone is again dramatically augmented when the cells are cultured in the presence of both reagents together. Analysis by DNA fragmentation also demonstrates that cells cultured alone or in combination with the above reagents exhibit the classical "ladder" of DNA fragmentation associated with apoptosis (Fig. 3*C*).

Combined Gliotoxin and TNF-α Treatment Does Not Cause Necrosis—Although our initial studies using trypan blue as a marker of plasma membrane integrity indicated that gliotoxin, both in the presence and absence of TNF-α, induced a purely apoptotic form of cell death, we felt it was important to validate this further by assessing necrosis in an independent manner using propidium iodide staining detected by flow cytometry. Fig. 3*D* shows the profile of neutrophils 4 h following treatment with gliotoxin (0.1 μg/ml) and TNF-α (10 ng/ml) where, despite apoptotic rates of 100%, almost all cells showed low fluorescence indicating that the cell membrane had remained intact. As a positive control, cells cultured initially with TNF-α and gliotoxin were then heat-treated (60 °C, 5 min) to ensure 100% necrosis. This resulted in a uniform and major increase in

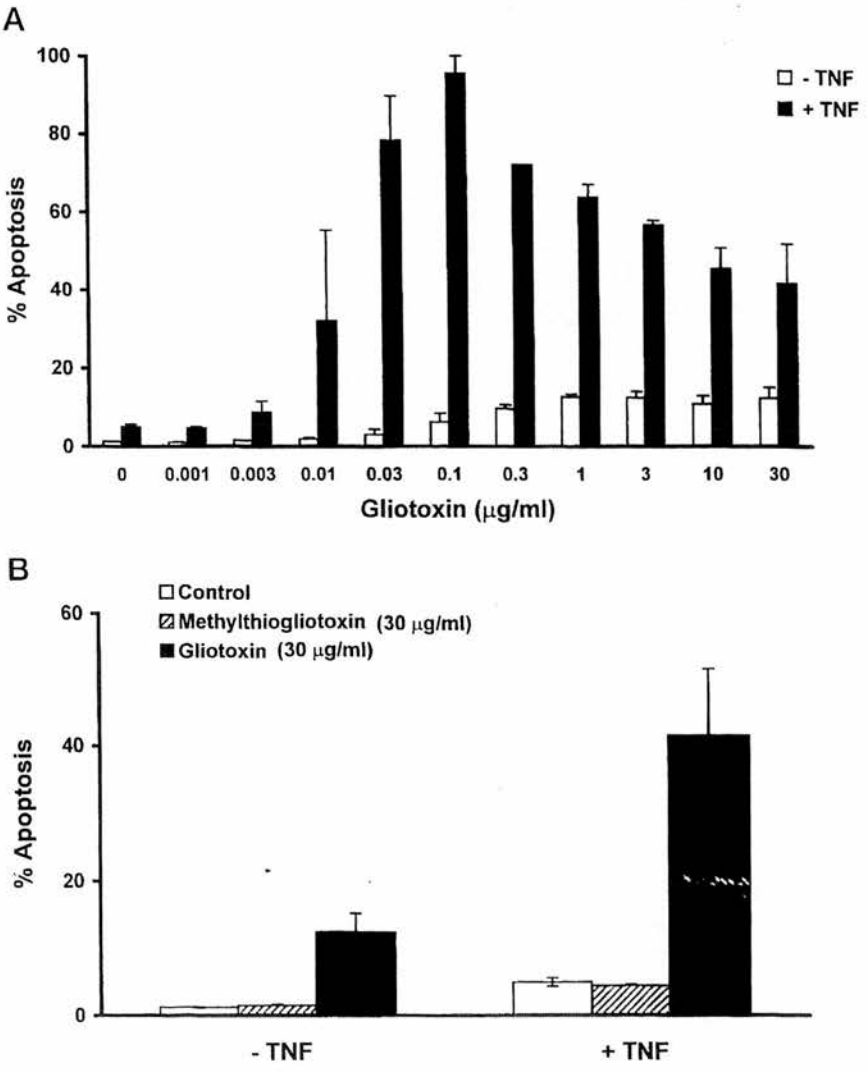


FIG. 2. **The effect of gliotoxin, methylthiogliotoxin, and TNF-α on human neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with the indicated concentrations of gliotoxin, plus or minus TNF-α. After 2 h of culture, the cells were resuspended and cytocentrifuge preparations made. These were fixed and stained, and apoptosis was assessed morphologically. *A*, represents the effect of gliotoxin (0.001–30 μg/ml) on TNF-α (10 ng/ml)-induced neutrophil apoptosis. *B*, represents the effect of methylthiogliotoxin (30 μg/ml) or gliotoxin (30 μg/ml), plus or minus TNF-α (10 ng/ml). All values represent mean ± S.E. of $n = 3$ experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.

propidium iodide staining (Fig. 3*D*). These data coincide completely with the results obtained with trypan blue staining and confirm that these cells had undergone apoptotic cell death and were not necrotic.

Gliotoxin Inhibits the Survival Effect of LPS—To investigate whether gliotoxin could modulate the effects of LPS on the rate of neutrophil apoptosis we performed a series of experiments where neutrophils were cultured for 2, 3, 4, and 20 h in the presence of LPS, gliotoxin, and a combination of LPS plus gliotoxin and apoptosis assessed morphologically (Fig. 4). As reported previously (7) LPS caused an inhibition of neutrophil apoptosis at 20 h when compared with control cells. Interestingly, the suppressive effect of LPS was prevented by the strong pro-apoptotic effect of gliotoxin. In addition, unlike co-culture of gliotoxin plus TNF-α, no synergistic induction of apoptosis was observed when LPS was cultured in the presence of gliotoxin (Fig. 4).

Gliotoxin Unmasks the Ability of TNF-α to Induce Eosinophil Apoptosis—To explore whether the pro-apoptotic effect of gliotoxin was restricted to neutrophils, a similar set of experiments were performed using human peripheral blood eosinophils isolated from mildly atopic individuals. While these cells display a similar capacity to undergo constitutive apoptosis when aged *in vitro*, this process is much slower than that observed for the neutrophil and is differentially regulated being, for example, stimulated rather than inhibited by corticosteroids (9). We therefore investigated the effect of gliotoxin on eosinophil apoptosis at 4 h in the presence and absence of TNF-α. The results, shown in Fig. 5, *A* and *B*, demonstrated that gliotoxin caused a similar induction of apoptosis in eosinophils ($EC_{50} = 0.37 \pm 0.22$ μg/ml) and caused a synergistic increase in the rate of apoptosis when the cells were co-cultured with TNF-α. This latter observation was all the more striking since in eosinophils, TNF-α treatment alone had no effect on the rate of apoptosis (Fig. 5*B* and data not shown). Hence, almost 100% apoptosis was observed using a gliotoxin concentration of 0.1 μg/ml plus TNF-α at a time point of 4 h. In comparison, eosinophils cultured in the absence of gliotoxin would normally show only 40% apoptosis at a 40-h time point (2, 36). Necrosis in these cells was <2% and the inactive analogue of gliotoxin had no effect on either the constitutive rate of apoptosis alone or in conjunction with TNF-α (data not shown). As with the neutrophils, eosinophils demonstrated classic apoptotic morphology when treated with these agents.

Gliotoxin Causes Inhibition of an Inducible Isoform of NF-κB—Recent studies have indicated that NF-κB may play an important role in regulating the rate of apoptosis in certain transformed cells (17, 18). Hence, because gliotoxin has been reported to act as a specific inhibitor of NF-κB (28) experiments were designed to identify and characterize the expression of this transcription factor in human neutrophils and determine if gliotoxin could inhibit such activity. Preliminary time course data established 90 min as the optimal time to examine basal, gliotoxin, and TNF-α regulated NF-κB activity in these cells

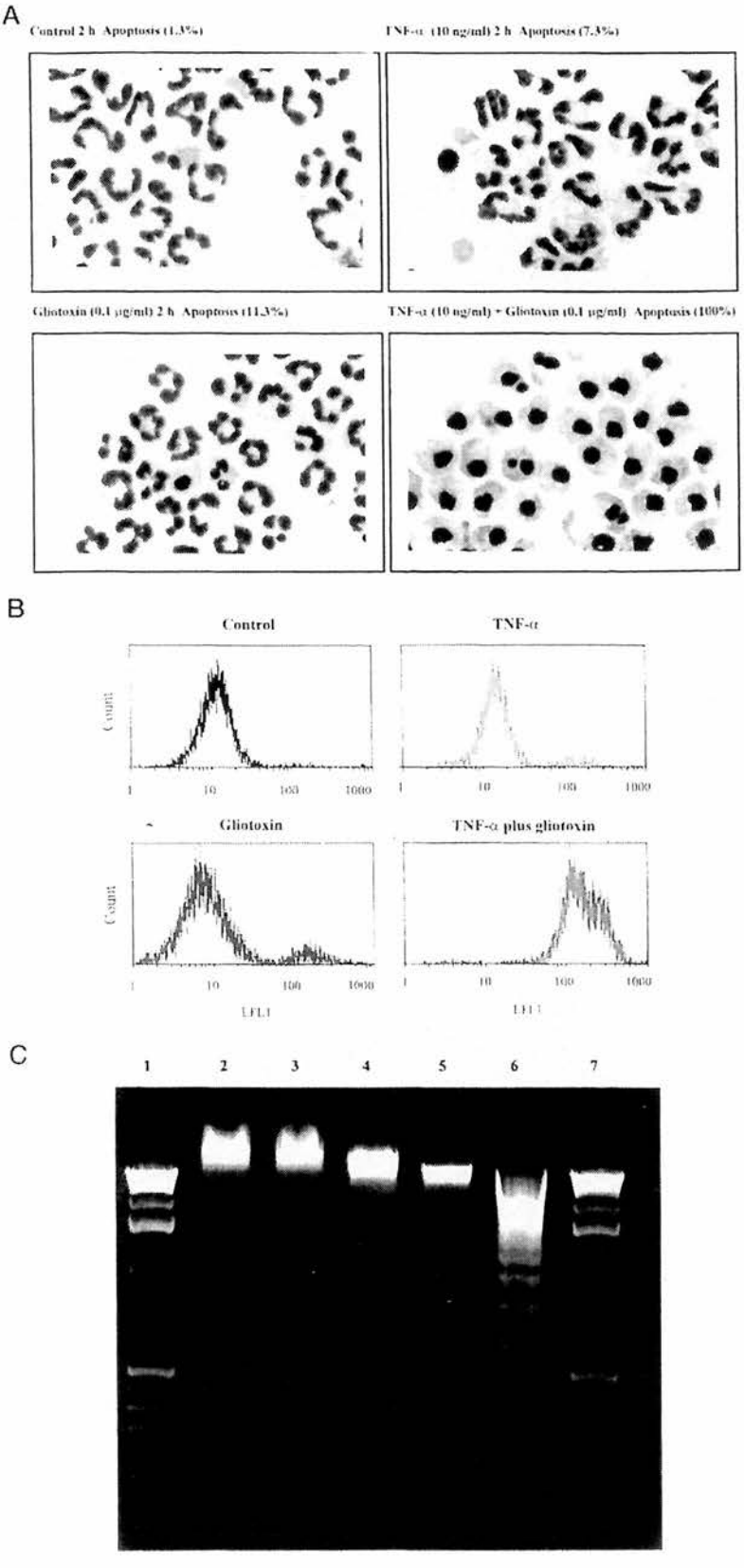


FIG. 3. **The effect of gliotoxin and TNF-α on neutrophil morphology, phosphatidylserine expression, and cell membrane integrity.** *A*, cytocentrifuge preparations of human neutrophils incubated for 2 h in Iscove's DMEM at 37 °C containing 10% autologous serum alone (*control*), plus TNF-α, plus gliotoxin, or with both TNF-α and gliotoxin at the concentrations shown. Neutrophils treated with both reagents all clearly show apoptotic morphology. *B*, after 2 h in culture at 37 °C, cells treated with Iscove's DMEM, TNF-α (10 ng/ml), gliotoxin (0.1 μg/ml), or with both TNF-α plus gliotoxin were resuspended and incubated with fluorescein isothiocyanate-labeled recombinant human annexin V to determine phosphatidylserine expression. The cells were then fixed and analyzed using an EPICS Profile II. Mean fluorescence values are shown for a minimum of 5,000 cells for each condition. *C*, DNA fragmentation in human neutrophils treated with TNF-α (10 ng/ml), gliotoxin (0.1 μg/ml), or both reagents together for 2 h. DNA was prepared as detailed under "Experimental Procedures"; *lane 1*, DNA marker (1-kilobase ladder); *lane 2*, freshly isolated neutrophils; *lane 3*, control; *lane 4*, TNF-α; *lane 5*, gliotoxin, 2 h; *lane 6*, co-culture with gliotoxin and TNF-α; *lane 7*, DNA marker (1-kilobase ladder). *D*, to assess cell membrane integrity, cells treated with both TNF-α (10 ng/ml) and gliotoxin (0.1 μg/ml) for 4 h, which induced 100% apoptosis even by 2 h, were resuspended and incubated with propidium iodide, fixed, and analyzed using an EPICS Profile II (*solid line*). An aliquot of cells from this preparation was heated as indicated under "Experimental Procedures" to produce 100% necrosis (*dotted line*). Mean fluorescence values are shown for a minimum of 5,000 cells for each condition.

(data not shown). Of note, this time point also coincided with the onset of the biologically observable effect of gliotoxin. As shown in Fig. 6, *A–C*, NF-κB EMSAs performed on neutrophil nuclear extracts indicated the presence of 3 discrete bands in these gels. To ascertain which of these bands were specifically NF-κB, an excess of unlabeled probe was included in the labeling reaction to displace specific binding; as shown in Fig. 6*C*, two NF-κB bands were identified and designated A and B.

In both TNF-α (10 ng/ml, 0–90 min) and LPS (1 μg/ml, 0–120 min) treated cells, no change in the intensity of band B was observed (Fig. 5, *A* and *B*, and data not shown). This, together with its strong expression in freshly prepared untreated neu-

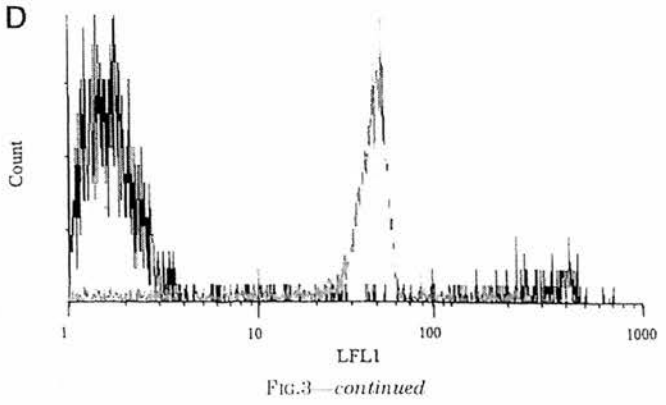


FIG. 3—*continued*

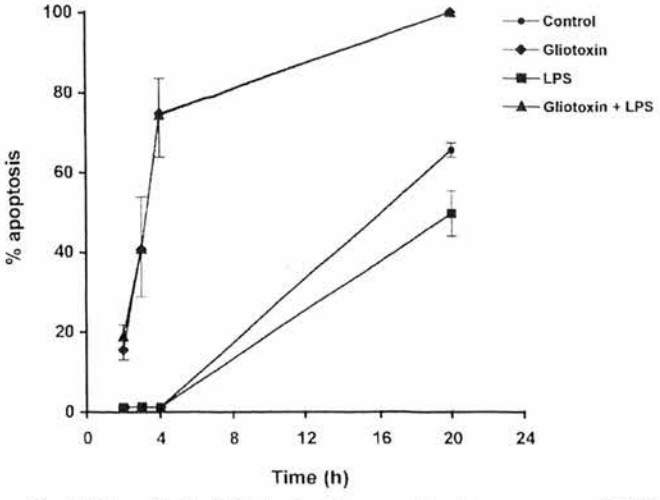
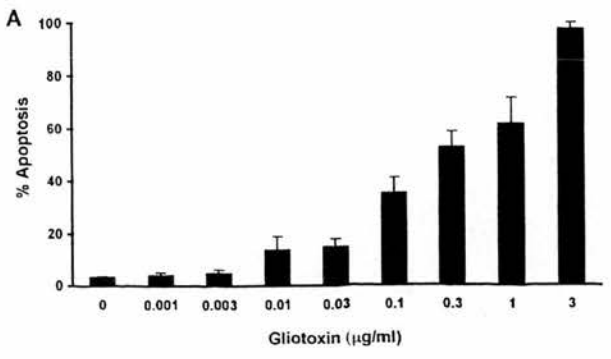


FIG. 4. **The effect of gliotoxin alone and in the presence of LPS on human neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured in Iscove's DMEM at 37 °C containing 10% autologous serum alone and treated with gliotoxin (0.1 μg/ml), with or without LPS (1 μg/ml). At the time periods indicated, the cells were resuspended and cytocentrifuge preparation made. These were fixed and stained, and apoptosis was assessed morphologically. All values represent mean ± S.E. of n = 3–5 experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.



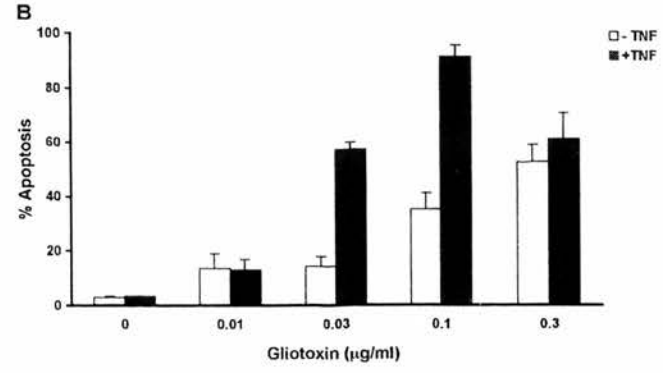


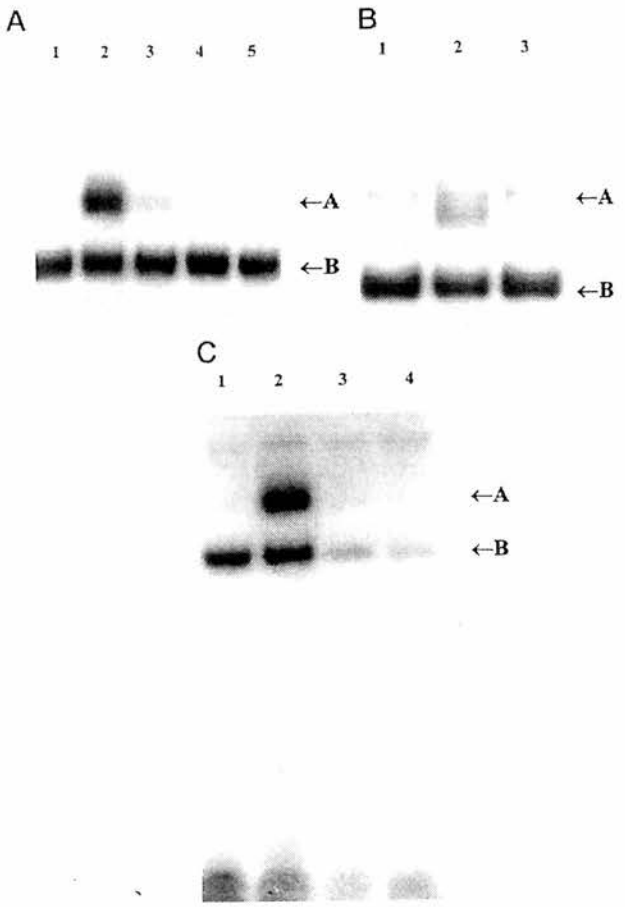
FIG. 5. **The effect of gliotoxin alone and in the presence of TNF-α on human eosinophil apoptosis.** Human eosinophils (2×10^6/ml) were cultured in Iscove's DMEM at 37 °C containing 10% autologous serum alone and treated with the indicated concentrations of gliotoxin with or without TNF-α. At the time periods indicated, the cells were resuspended and cytocentrifuge preparation made. These were fixed and stained, and apoptosis was assessed morphologically. *A*, represents the effects of gliotoxin (0–3 μg/ml) on eosinophil apoptosis after 4 h of culture. *B*, represents the effects gliotoxin (0–0.3 μg/ml) with or without TNF-α (10 ng/ml) on eosinophil apoptosis after a 4-h culture. All values represent mean ± S.E. of n = 3 experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.

trophils suggests that this band represents a form of constitutively active NF-κB. However, band A was markedly up-regulated by TNF-α (Fig. 6, *A* and *C*), and as shown in Fig. 6*A*, gliotoxin caused both a concentration-dependent inhibition of this NF-κB activity and abolished the TNF-α stimulated increase in this band. As shown in Fig. 6*D*, densiometric analysis of these data confirmed that co-treatment of neutrophils with TNF-α and gliotoxin at a maximal effective functional concentration of 0.1 μg/ml (Fig. 2*A*) inhibited this band more than treatment with gliotoxin alone. The results in Fig. 6, *A* and *D*, confirm that the basal level of NF-κB activity in control samples at 90 min was inhibited by gliotoxin treatment; a finding that was observed at all other time points tested.

Further evidence that strongly supports the suggestion that NF-κB inhibition is linked to the induction of neutrophil apoptosis is provided by the fact that the cell permeable NF-κB inhibitory peptide, SN50 (37), also increased the rate of constitutive neutrophil apoptosis despite the fact that less than 5% of the peptide reportedly enters the cell (37). Hence at 6 h, the SN50 peptide increased neutrophil apoptosis from 4.7 ± 1.2 to 15.0 ± 3.2% (n = 3; $p < 0.05$), whereas the less active peptide SN50M only increased apoptosis from 4.7 ± 1.2 to 5.6 ± 0.8% (n = 3). Similar effects on neutrophil apoptosis were seen at 20 h and eosinophil apoptosis at 20 and 40 h (Table I and data not shown). Other agents known to inhibit NF-κB similarly induced neutrophil apoptosis. The proteasome inhibitor, MG-132 (38) and the NF-κB inhibitor curcumin (39) caused a time-dependent induction of neutrophil apoptosis (Fig. 7*A*). PDTC, that acts as both a radical scavenger and inhibitor of NF-κB activation (40), also caused a significant induction of apoptosis when cultured with neutrophils for 20 h (Fig. 7*B*). Furthermore, treating neutrophils with LPS (100 ng/ml, 20 min) which we have previously reported to induce a profound inhibition of neutrophil apoptosis (7) was found to cause the appearance of this inducible isoform of NF-κB (Fig. 6*B*); and this induction could be inhibited by gliotoxin (0.1 μg/ml).

Induction of Apoptosis by Gliotoxin Is Dependent on Activation of the Caspase-cascade Pathway—We have recently demonstrated that the early pro-apoptotic effects of TNF-α in human neutrophils requires activation of both TNF-55 and TNF-75 receptor subtypes and thereby differs significantly from the priming effect of TNF-α which is signaled via the TNF-p55 receptor alone (10). To determine whether the pro-apoptotic effects of gliotoxin and the marked synergism displayed by TNF-α and gliotoxin were mediated via activation of the caspase pathway, we co-incubated neutrophils with TNF-α, gliotoxin, and zVAD-fmk. At 2 h, zVAD-fmk completely inhibited the increase in apoptosis induced by gliotoxin, TNF-α and by both factors together (Fig. 8*A*). This demonstrates that apoptosis induced by both factors alone, or together, is dependent on caspase activation.

Gliotoxin May Enhance TNF-α-induced Apoptosis by Inhibiting Production of a Survival Factor—Taken together, the



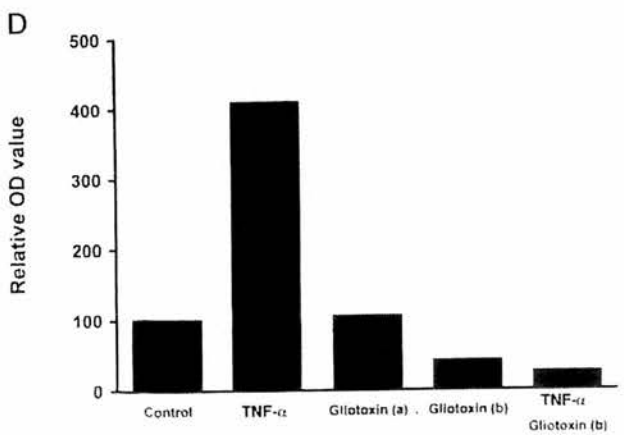


FIG. 6. **Effect of gliotoxin and TNF-α on NF-κB mobilization.** *A*, EMSA of nuclear extracts from neutrophils treated with control buffer; *lane 1*, TNF-α (10 ng/ml); *lane 2*, gliotoxin (1 μg/ml); *lane 3*, gliotoxin (0.1 μg/ml); *lane 4*, and TNF-α (10 ng/ml) plus gliotoxin (0.1 μg/ml); *lane 5*, for 90 min at 37 °C. *B*, EMSA showing the up-regulation of the inducible isoform (*band A*) by LPS (a known inhibitor of neutrophil apoptosis) after 20 min culture: *lane 1*, control; *lane 2*, LPS (100 ng/ml); and *lane 3*, LPS (100 ng/ml) plus gliotoxin (0.1 μg/ml). *C*, EMSA showing displacement of specific NF-κB bands by excess cold oligonucleotide probe; *lane 1*, control; *lane 2*, TNF-α (10 ng/ml); *lane 3*, TNF-α (10 ng/ml) plus 50-fold excess cold oligonucleotide; *lane 4*, TNF-α (10 ng/ml) plus 100-fold excess cold oligonucleotide. Only the bands marked A and B are specific. *D*, densiometry scanning of band A from the EMSA shown in *A*. This shows the reduction of an inducible isoform of NF-κB by gliotoxin (*a* 1 μg/ml; *b* 0.1 μg/ml), and further inhibition by co-treatment with TNF-α plus gliotoxin (b).

above results suggest that activation of an inducible form of NF-κB may inhibit or restrain the pro-apoptotic effects of TNF-α which are mediated by the parallel activation of the

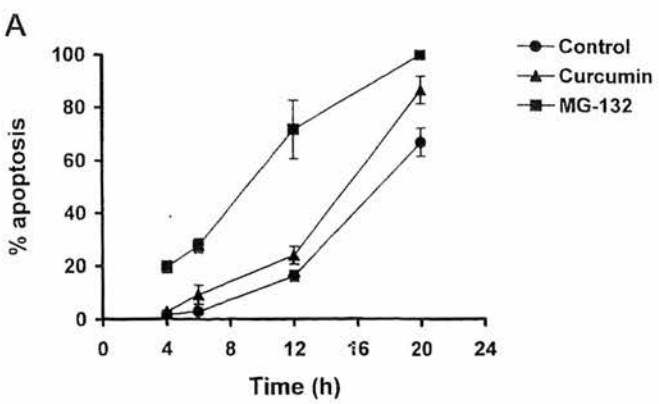
TABLE I

The effect of NF-κB inhibitory peptides on human neutrophil apoptosis

Human neutrophils (5×10^6/ml) were resuspended in Iscove's DMEM without autologous serum and treated with control buffer, SN50 (100 μg/ml), and SN50M (100 μg/ml). After 15 min at 37 °C, 10% autologous serum (final concentration) was added, and after 6 and 20 h of culture, the cells were resuspended and cytocentrifuge preparations made. These were fixed and stained, and apoptosis was assessed morphologically. All values are from n = three separate experiments, each performed in triplicate.

Treatment	Time	Mean	S.E.
		% apoptosis	
Control (buffer)	6h	4.7	1.2
SN50 (active)	6h	15.0[a]	3.3
SN50M (less-active)	6h	6.8	5.6
Control (buffer)	20h	58.9	7.7
SN50 (active)	20h	81.4[a]	2.7
SN50M (less-active)	20h	70.3	8.2

[a] Represents significance differences ($p < 0.05$) from the appropriate control.



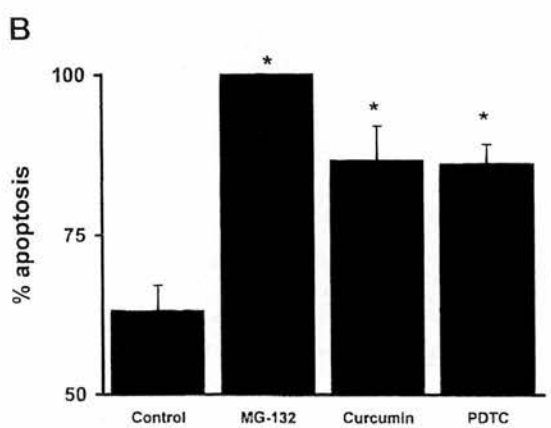


FIG. 7. **Effect of other NF-kB inhibitors on neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured at 37 °C in Iscove's DMEM containing 10% autologous serum and treated with the indicated reagent. *A*, neutrophils were treated with MG 132 (20 μM) and curcumin (20 μM) at the time periods indicated; *B*, neutrophils were treated with MG-132 (100 μM), curcumin (20 μM), and PDTC (300 μM) for 20 h. After incubation, the cells were resuspended and cytocentrifuge preparations made. These were fixed and stained, and apoptosis was assessed morphologically. All values represent mean ± S.E. of n = three to six experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.

caspase pathway. The possibility that this most likely reflects the production of a protein or proteins which act to suppress the activation of the caspase pathway and thus protect granulocytes from the cytotoxic effects of this cytokine was investigated by incubating neutrophils with TNF-α and cyclohexi-

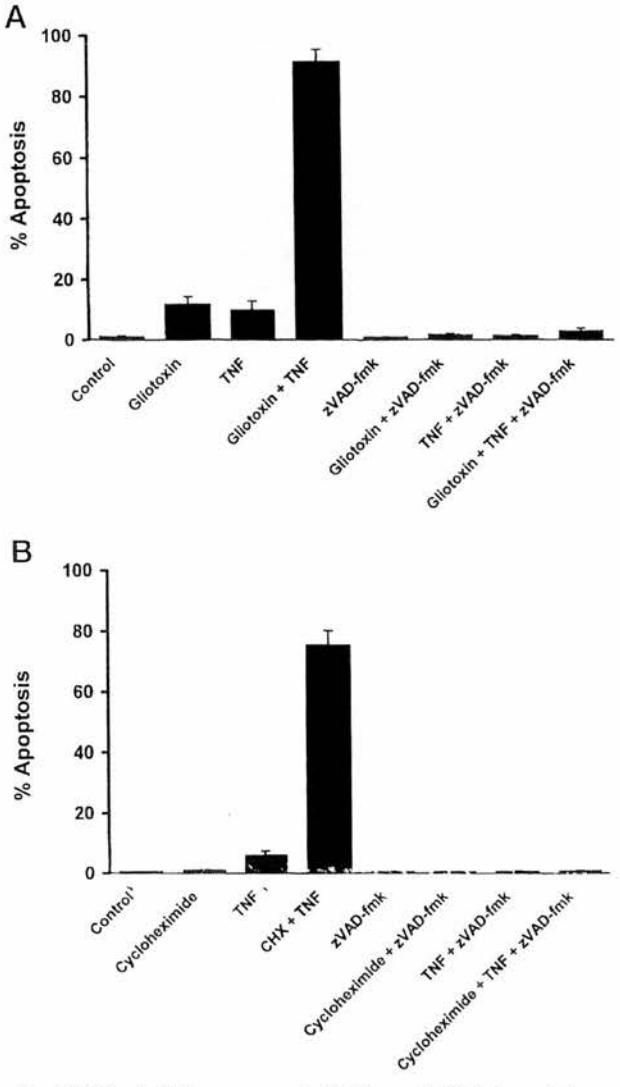


FIG. 8. **Effect of the caspase inhibitor, zVAD-fmk, and the protein synthesis inhibitor, cycloheximide, on gliotoxin, TNF-α, or gliotoxin plus TNF-α-induced human neutrophil apoptosis.** Human neutrophils (5×10^6/ml) were cultured in DMEM containing 10% autologous serum and treated with the indicated reagents. After 2 h of culture, the cells were resuspended and cytocentrifuge preparation made. These were fixed and stained, and apoptosis was assessed morphologically. *A*, represents the effects of zVAD-fmk (100 μM) on gliotoxin (0.1 μg/ml)-, TNF-α (10 ng/ml)-, or gliotoxin plus TNF-α-induced neutrophil apoptosis. *B*, represents the effects of cycloheximide (5 μM) on gliotoxin (0.1 μg/ml)-, TNF-α (10 ng/ml)-, or gliotoxin plus TNF-α-induced neutrophil apoptosis. All values represent mean ± S.E. of n = three experiments, each performed in triplicate. Where not shown, S.E. values are less than 2% of the mean.

mide, a protein synthesis inhibitor. As illustrated in Fig. 8*B*, cycloheximide used specifically at a concentration (5 μM) that alone had almost no effect on neutrophil apoptosis at 2 h, caused a synergistic increase in the level of apoptosis when neutrophils were co-cultured with TNF-α. This supports the view that TNF-α treatment indeed results in the generation of a survival protein that protects these cells from the TNF receptor-caspase-dependent induction of apoptosis by TNF-α.

DISCUSSION

We have demonstrated that gliotoxin, but not its inactive derivative methylthiogliotoxin, (*a*) induces a direct time- and concentration-dependent increase in the rate of constitutive apoptosis in both neutrophils and eosinophils, (*b*) enhances the pro-apoptotic effect of TNF-α in neutrophils, and (*c*) reveals the cytotoxic effects of TNF-α in eosinophils. In these studies extreme care was taken to ensure that gliotoxin, at all time points and concentrations studied, was non-toxic and caused genuine apoptosis that was indistinguishable from later constitutive apoptosis. The similar effects of gliotoxin in both neutrophils and eosinophils and the same concentration of gliotoxin (0.1 μg/ml) required for maximal enhancement of the pro-apoptotic effects of TNF-α, suggests that an identical underlying mechanism is regulating the induction of cell death in both these cell types. However, although inhibition of basal NF-κB activity may be involved in neutrophil apoptosis when induced by gliotoxin alone (Fig. 1) since gliotoxin appears to block basal levels of NF-κB activity (Fig. 6, *A* and *D*), only the expression of the inducible NF-κB isoform is down-regulated before the onset of cell death driven by the combined effects of TNF-α and gliotoxin (Fig. 6). Even in control neutrophils incubated for 20 h, where the constitutive rate of apoptosis is approximately 70%, the density of the constitutive NF-κB band was unaffected (data not shown). These differences in the inducible and constitutive forms of NF-κB most likely reflects differential regulation of activation, for example, by the involvement of different isoforms of the inhibitory IκB subunit, or that the constitutively active NF-κB is formed from a different set of dimers from the classical RelA/p50 heterodimer. It has recently been demonstrated that neutrophils contain c-Rel, p50, and p105 (the p50 precursor protein) as well as Rel-A (22, 41). The inducible band we observed has also been reported to be up-regulated in neutrophils by phagocytosis of IgG opsonized yeast particles (42), and has been shown to consist mainly of Rel-A/p50 heterodimers and possibly a small amount of c-Rel (42). In that study, phagocytosis of these particles did not affect the activity of the constitutive complex. In addition, it has recently been reported that NF-κB becomes activated, via a mechanisms not involving oxidant generation, when neutrophils phagocytose bacteria (43).

Because NF-κB is also activated by certain pro-apoptotic stimuli such as TNF-α, this transcription factor has been considered as a possible regulator of cell death. Hence, in some T cell clones, activation of NF-κB appears to correlate with the onset of apoptosis (18). However, NF-κB activation has clearly been shown to be anti-apoptotic in HT 1080 fibrosarcoma cells (21) and TNF-α induced NF-κB activation prevents cell death in HeLa and MCF7 cells (44). Here we show for the first time that in a non-transformed cell namely the neutrophil, inhibition of an inducible form of NF-κB is related to the induction of apoptosis.

Several mechanisms, aside from NF-κB inhibition, have been proposed for the pro-apoptotic actions of gliotoxin in other cells. Sutton *et al.* (45) have shown that although this fungal metabolite did not affect intracellular calcium levels, there was a correlation between increases in cAMP levels and apoptosis in gliotoxin-treated splenocytes. However, we and others have previously demonstrated that agents that elevate intracellular cAMP inhibit apoptosis in both neutrophils and eosinophils (33, 46). It has also been suggested that protein kinase A-dependent phosphorylation of histone H3 correlates with gliotoxin-induced apoptosis in thymocytes (47), but again in neutrophils, activation of protein kinase A inhibits apoptosis (33). Although gliotoxin has been reported to inhibit protein synthesis (48) it is highly unlikely that this mechanism is directly responsible for its pro-apoptotic effects: first, gliotoxin induces apoptosis in thymocytes whereas inhibition of protein synthesis by cycloheximide inhibits thymocyte apoptosis. Second, since NF-κB activation is involved in the control of multiple genes, many of which encode for inflammatory mediator synthesis, inactivation of NF-κB would therefore be expected to inhibit protein

synthesis. Third, while protein synthesis inhibitors do up-regulate the rate of constitutive cell death in granulocytes, the kinetics of this response are very different to those observed with gliotoxin. For example, Whyte *et al.* (49) have reported that cycloheximide (50 μM) and actinomycin D (1 μM) induce apoptosis in approximately 30% of neutrophils by 6 h. In our experiments, gliotoxin (0.1 μg/ml), induces a rate of almost twice this, whereas 1.0 μg/ml gliotoxin induced 100% neutrophil apoptosis by this time point (see Fig. 1*A*). Likewise, our own results with cycloheximide indicate that protein synthesis inhibition alone does not affect the rate of neutrophil apoptosis at 2 h whereas gliotoxin alone produced almost 15% apoptosis over this period (Figs. 1*A* and 8*B*). While gliotoxin inhibits NF-κB, cycloheximide and actinomycin D have been shown in several systems to activate this transcription factor (50, 51). Although both cycloheximide and gliotoxin give a similar synergistic pro-apoptotic response with TNF-α, this suggests that different mechanisms must be involved. However, while gliotoxin may prevent synthesis of a protective protein inducible by NF-κB activation, cycloheximide would also preclude synthesis of such a protein so that in both cases the cells would be sensitive to the pro-apoptotic effects of TNF-α. It is of interest to note that granulocytes do have the capacity to synthesize proteins, albeit in a limited capacity (49). We believe that this synthetic capacity will be directed toward resolution of the inflammatory response with the generation of protein(s) that affect the apoptotic program of inflammatory cells.

Our results indicate that the inducible isoform of NF-κB disappears from the gliotoxin-treated granulocyte nucleus just before the onset of stimulated apoptosis. The possibility that these events are causally related is supported by the following observations: (i) the synthetic cell-permeable peptide SN50 (37), a known inhibitor of NF-κB, also induces apoptosis in neutrophils and eosinophils; (ii) other agents that inhibit NF-kB activation, namely PDTC and curcumin as well as the proteasome inhibitor MG-132 also cause an induction of granulocyte apoptosis; (iii) the kinetics for gliotoxin-mediated inhibition of NF-κB match those for the onset of induction of apoptosis; (iv) LPS which stimulates NF-κB activity prolongs neutrophil and eosinophil survival; and (v) that gliotoxin sensitizes both neutrophils and eosinophils to the pro-apoptotic effects of TNF-α. Indeed, our studies provide the first plausible explanation for the modest and temporally constrained apoptotic response of neutrophils to TNF-α and the observation that pretreatment with LPS, PAF or granulocyte/macrophage-colony stimulating factor, abolishes the cytotoxic effect of this cytokine (10). Indeed, this latter point is of particular relevance when investigating the pro-apoptotic effect of TNF-α in neutrophils since pre-treatment of these cells causes a rapid decrease of both TNF-α receptors subtypes from the surface membrane (10). This phenomenon, together with the fact that the effects of SN50 are, at best, modest due to limited access of the peptide to its intracellular target (37) and the requirement for pretreatment with the peptide, precluded accurate assessment of SN50 on TNF-α induced apoptosis in neutrophils.

When neutrophils were co-cultured with LPS and gliotoxin, gliotoxin failed to render LPS pro-apoptotic despite the fact that LPS induced survival was inhibited by gliotoxin (Fig. 4). These results suggest that LPS does not trigger a death pathway in neutrophils but stimulates a NF-κB-mediated survival pathway *i.e.* when NF-κB activation is blocked, LPS is no longer capable of delaying apoptosis. The precise intracellular mechanisms by which the NF-κB inhibitors used in this study induce apoptosis are unknown and is the subject of further investigation. For example, it would be of interest to perform an in-depth analyses of the effect of gliotoxin and the other agents on the degradation of the inhibitory subunit IκB, especially since Pahl *et al.* (28) reported that gliotoxin appeared to prevent IκB degradation rather than mediate its effect at the level of DNA binding.

In a number of immune cells NF-κB activation by agents such as TNF-α has been shown to play a central role in regulating the genes for inflammatory cytokines such as granulocyte/macrophage-colony stimulating factor and TNF-α itself (14). The importance of this response *in vivo* is that many of these factors inhibit granulocyte apoptosis and may therefore delay inflammatory resolution by enhancing the longevity of these cells. Indeed, a positive-feedback loop may exist since many of these inflammatory mediators which protect against apoptosis in neutrophils and eosinophils also activate NF-κB (7). Conversely, we have recently shown that NO, a known inhibitor of NF-κB (52, 53), is a potent inducer of apoptosis in neutrophils (54). Our current results suggest that the activation of an inducible form of NF-κB represents a powerful survival mechanism in granulocytes, and that when this pathway is inhibited, in both neutrophils and eosinophils, these cells undergo a greatly augmented rate of apoptotic cell death. It is possible that NF-κB performs a similar function in other cell types that undergo apoptosis in response to gliotoxin.

Enhanced cytotoxic responses to TNF-α have also been demonstrated in cells where NF-κB is genetically deficient or inactivated (19–21) and hepatocytes from Rel-A null mice are known to undergo apoptosis causing death *in utero* (55). Embryonic fibroblasts and macrophages from Rel-A-deficient mice also showed dramatic loss of viability when treated with TNF-α leading to the suggestion that Rel-A regulates a protective mechanism against the cytotoxic effects of TNF-α. It would be of interest to investigate the effects of TNF-α and gliotoxin on granulocytes isolated from mice deficient in Rel A; it would be reasonable to predict that TNF-α will induce a rapid cell death and gliotoxin and other NF-κB inhibitors would not have a dramatic effect on the rate of granulocyte apoptosis. Although our experiments indicate that gliotoxin does not inhibit the constitutive form of NF-κB, at least at early time points, it does inhibit the activation of an inducible isoform of NF-κB, which most likely consists of heterodimers containing the Rel-A/p65 protein and therefore could perform a similar anti-apoptotic function in neutrophils and eosinophils. While in our hands TNF-α does not produce significant cytotoxic effects in eosinophils, co-treatment with gliotoxin caused these cells to become highly responsive to this cytokine producing greatly increased levels of apoptosis. This suggests that both of these inflammatory cell types could be stimulated to undergo apoptosis and hence be cleared rapidly by phagocytes at sites of inflammation if activation of the inducible NF-κB isoform were inhibited.

The mechanism whereby inactivation of NF-κB induces granulocyte apoptosis and increases the cytotoxic response to TNF-α is currently unclear. Since gliotoxin and TNF-α driven apoptosis are both inhibited by the caspase-inhibitor zVAD-fmk, this, together with the synergy for apoptosis observed with these agents, implies that NF-κB or an NF-κB regulated step influences granulocyte apoptosis at an intermediate step between the TNF-α receptor and caspase activation. The possibility that NF-κB controls the transcriptional activity of a gene(s) which induces the synthesis of survival proteins is supported by the observation that cycloheximide also increases apoptosis in granulocytes (49). This suggests a strong link between inducible NF-κB activation and the control of TNF-α-induced apoptosis, possibly via the production of a protein inhibitor of this pathway. Indeed, as we have shown, protein synthesis inhibition enhances the pro-apoptotic effect of TNF-α as early as 2 h of culture. Indeed, one possible candidate for this

protein has already been suggested: A20, a protein induced by TNF-α activation of NF-κB (56, 57), has been shown to protect against TNF-α induced cell death by acting at the level of the TNF-α receptor-associated proteins TRAF-1 and TRAF2 (58). Although A20 has yet to be demonstrated in neutrophils or eosinophils, this would represent an attractive candidate protein to fulfill such a role.

The ability of gliotoxin to enhance the cytotoxic effects of TNF-α and itself produce a rapid onset of apoptosis in inflammatory cells such as neutrophils and eosinophils may suggest NF-κB inhibition as a logical therapeutic target in the treatment of inflammatory conditions. In a rat model of lung inflammation, suppression of NF-κB activity has already been shown to block the development of neutrophil lung inflammation by inhibiting the synthesis of chemotaxins (59). Our results suggest that NF-κB inhibition may also be of benefit in enhancing the resolution of inflammation by allowing a more rapid clearance of granulocytes. We therefore propose that granulocyte apoptosis is regulated by an inducible form of the transcription factor NF-κB and suggest that inhibition of this transcription factor may be exploited for therapeutic benefit in inflammatory conditions where granulocytes play a prominent role.

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Inhibition of NF-κB by a cell permeable form of IκBα induces apoptosis in human eosinophils.

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Running title; TAT- IκBα induces eosinophil apoptosis

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Abstract

An 11 amino acid HIV-TAT peptide can deliver target proteins into a variety of cells in a receptor independent manner. To generate a highly specific inhibitor of the transcription factor NF-κB, we have fused the TAT-peptide to a version of IκBα that is resistant to signal-induced degradation. TAT-IκBα(S32A, S36A) inhibited NF-κB dependent transcription in HeLa and A549 cells by retaining NF-κB p65 in the cytoplasm. Introduction of TAT-IκBα(S32A, S36A) into human eosinophils inhibited the nuclear translocation of NF-κB and induced apoptosis. Thus, continuous NF-κB dependent transcription is important for eosinophil survival. While eosinophils are normally refractive to standard methods of gene delivery, the ability of TAT fusion proteins to be taken up by these cells should enable a detailed molecular analysis of survival pathways in these cells.

1. Introduction

Excessive eosinophil recruitment and activation at inflammatory sites is likely to cause surrounding tissue damage by liberation of their toxic granules contents. Toxic granule proteins are important for defence against parasites, but if they are released in the host tissue when eosinophils are inappropriately activated, they will contribute to the progression of inflammatory diseases such as asthma. Thus levels of eosinophilic toxic granules components such as major basic protein, eosinophil cationic protein, eosinophils peroxidase and cysteinyl leukotrienes have been found in significantly increased amounts in the airways of asthmatic patients and animal models suggesting that they may be responsible for the airway tissue damage and remodelling [1-3]. An additional function of eosinophils is the production of pro-inflammatory cytokines including IL-1, IL-4, IL-6, IL-8, TNFα, GM-CSF and IL-5 [4]. Synthesis of these proteins in eosinophils is dependent on activation of intracellular signalling cascades and key transcription factors. Some of these proteins trigger the activation of inflammatory cells, including eosinophils thereby contributing to an amplification of the inflammatory response and disease progression. Therefore, effective removal of eosinophils from inflammatory sites may be a key factor for successful resolution of the inflammatory process. As previously shown from our study [5, 6] and others [7, 8] NF-κB is a key regulator of eosinophil activation and survival.

NF-κB is a family of transcription factors that regulates survival and inflammatory responses [9, 10]. The NF-κB family contains the proteins p65/*Rel* A, c-*Rel*, *Rel* B, p50/

NF-κB1, and p52/ NF-κB2 in various combinations to form the active transcriptional dimer. NF-κB transcriptional activity is controlled by the IκB family of inhibitory proteins which contain ankyrin repeat domains (ARD) and share the function of retaining NF-κB in the cytoplasm. IκBα is a 37kDa protein that binds to NF-κB, retaining it in the cytoplasm and inhibiting DNA binding. The pro-inflammatory cytokines TNFα and IL-1β initiate a signalling cascade via their receptors on the cell surface and activate the IκB kinases consisting of subunits, IKK-1 (or IKK-α) and IKK-2 (or IKK-β), and a structual subunit IKKγ or NEMO. The activated kinase phosphorylates IκBα on serines 32 and 36 and phophorylated IκBα is selectively ubiquitinated on lysine 21 and 22 by an SCF E3 ubiquitin ligase contain βTrCP. Poly-ubiquitinated IκBα is degraded by the 26S proteasome [11] and the released NF-κB translocates to the nucleus where it activates transcriptional responsive genes. The pro-inflammatory cytokine TNFα can trigger at least two antagonistic pathways: a survival pathway via NF-κB transcriptional activation and a pro-apoptotic pathway via activation of caspase cascades. Thus inhibition of NF-κB un-masks the caspase-dependent pro-apoptotic properties of TNFα in many cells [12-14] including granulocytes [6].

The study of specific intracellular signalling pathways in eosinophils is technically challenging because they are refractory to many methods of direct molecular manipulation such as transfection, viral infection or microinjection. Recently an 11 amino acid HIV-TAT has been highlighted for its ability to deliver target proteins into a variety of cells in a receptor independent manner. It has been demonstrated that the arginine rich motif (ARM), corresponding to amino acids 47-57 of HIV-1 TAT (YGRKKRRQRRR)

could transduce materials such as antibodies and enzymes, nucleic acids and beads into cells in a receptor and transporter independent manner [15]. Furthermore linkage of TAT to proteins such as p27 [15], caspase3 [16], GTPase [17] and β-galactosidase [18] resulted in almost 100% transduction efficiency in 30 min of many cells types including granulocytes [19-21].

Here, we generated a TAT-IκBα fusion protein that is resistant to signal induced degradation and demonstrate that it is capable of inhibiting NF-κB dependent transcription in a variety of cell types. Introduction of TAT-IκBα(S32A, S36A) into human eosinophils inhibited the nuclear translocation of NF-κB and induced apoptosis, thus demonstrating the utility of this approach.

2. Materials and Methods

2.1. Cloning TAT-IκBα and GST-TAT

pcDNA IκBαWT (wild type, full length) and pcDNA S32A, S36AIκBα were described [23]. The sequence encoding an 11 amino acid TAT peptide (YGRKKRRQRRR) [15] inserted into pGEX2T (Pharmacia, New Jersey,USA). GST-TAT-IκBα and GST-TAT-IκBα (S32A, S36A) and GST-TAT were purified essentially as described [24].

2.2. Labelling TAT with FITC

Fluorescein 5-isothiocyanate (FITC) was incubated with TAT-IκBα, recombinant IκBα or GST-TAT (40-80μg per mg of protein) for 1 hour at room temperature then, overnight at 4°C in the dark. After coupling, protein was dialysed against buffer (10mM Tris, 150mM NaCl, pH8.2), at 4°C with four changes of the same buffer and the protein concentration determined as described [24].

2.3. Luciferase Assay

HeLa cells, 57A [25] containing an integrated NF-κB dependent luciferase reporter were maintained in 10% FCS in DMEM. Following treatment with TAT-IκBα or GST-TAT cells were exposed to TNFα to activate NF-κB and 6 hours later luciferase specific activity (RLU/mg) was determined described [25].

2.4. Other experimenal methods

Human eosinophils isolation, assessment of eosinophil apoptosis, statistical analysis, isolation of cytoplasmic and nucleus extract for Western Blotting and fluorescence

microscopy were as described [5], with the following modifications. The images of fluorescence microscopy were captured using Open Lab software (Improvision, Coventry, UK) and CoolSnap digital camera (Media Cybernetics, Silver Spring, MD, USA). To prepare cytoplasmic and nucleus extract for Western Blotting, $5x10^6$ eosinophils and $1x10^6$ A549 cells were used for each condition, and were analysed with anti-IκBα (Cell Signalling Technology, Manchester, UK) and anti-p65 antibodies (Santa Cruzu Biotechonology Inc, California, USA).

3. Results

3.1. Inhibition of NF-κB Activation by TAT-IκBα

The 11 amino-acid TAT peptide (YGRKKRRQRRR) can mediate uptake of coupled molecules into cells. Therefore, to generate a cell permeable molecule capable of specifically inhibiting NF-κB the TAT peptide was fused to IκBα, the natural inhibitor of NF-κB and IκBα (S32A, S36A) (figure 1A) that is resistant to signal induced degradation [26]. As a negative control the TAT peptide was also fused to GST. These proteins and recombinant IκBα lacking the TAT peptide were expressed in bacteria and purified as described [24]. MALDI- TOFF mass spectrometry confirmed that the proteins were of the predicted molecular mass (TAT-IκBα(S32A, S36A); 37570 a.m.u. and recombinant IκBα (rIκBα); 35471 a.m.u.) and SDS-PAGE followed by Coomassie blue staining indicated that they were essentially homogenous (figure 1B).

To determine the ability of the TAT-IκBα fusion to block NF-κB activation, recombinant proteins were applied to a HeLa cell line (57A) containing an integrated NF-κB luciferase reporter gene [25] and preincubated for 30 min prior to addition of TNFα. After a further 6 hours cells were harvested and luciferase activity was determined. TNFα alone induces a large (approximately 100-fold) activation of NF-κB dependent reporter activity and this was substantially inhibited by addition of TAT-IκBα(S32A, S36A), but not by GST-TAT or rIκBα (figure 1C). To compare the efficiency with which TAT-IκBα(S32A, S36A), and TAT-IκBα(WT) inhibit NF-κB activation, a range of concentrations of the proteins were preincubated with HeLa 57A prior to addition of

TNFα and reporter activities determined. While TAT-IκBα(WT) displays some inhibitory potential. It is clear that TAT-IκBα(S32A, S36A) is considerably more efficient at inhibiting NF-κB dependent transcription (figure 1D). Even at a low dose (0.03 μg/ml) TAT-IκBα(S32A, S36A) reduces TNF-α-activated luciferase activity by 61%. Therefore, TAT-IκBα(S32A, S36A) was chosen for further study.

3.2. Mechanism of Inhibition of NF-κB by TAT-IκBα

To investigate the mechanism by which TAT-IκBα inhibits signal induced activation of NF-κB, the alveolar epithelial cell line A549 was preincubated with TAT-IκBα(S32A, S36A) prior to addition of TNFα, cells were lysed and IκBα levels determined by Western Blotting (WB) analysis. In the absence of TAT-IκBα(S32A, S36A), TNFα induces the degradation of endogenous IκBα (figure 2A, lanes 1 and 2). Exogenous TAT-IκBα(S32A, S36A) is resistant to TNFα induced degradation, but does not protect endogenous IκBα from signal induced degradation (figure 2A, lanes 3 and 4). GST-TAT does not influence TNFα induced degradation of IκBα (figure 2A, lanes 5 and 6).

To determine the influence of TAT-IκBα(S32A, S36A) on TNFα-induced nuclear translocation of NF-κB, cells were pretreated with TAT fusion protein and the subcellular localisation of NF-κB determined by immuofluorescence microscopy.

In untreated cells, p65 is predominantly cytoplasmic but after treatment with TNFα translocates to the nucleus (figure 2B, left panels). However, pre-treatment of cells with

TAT-IκBα(S32A, S36A) blocks TNFα induced nuclear translocation of NF-κB p65. Pre-treatment of cells with GST-TAT did not influence TNFα induced nuclear translocation of NF-κB p65 (figure 2B, right panels). Thus, TAT-IκBα(S32A, S36A) does not inhibits TNFα-induced degradation of endogenous IκBα but blocks nuclear translocation of the released NF-κB.

3.3. TAT-IκBα Transduction Into Eosinophils

Unlike established cell lines, eosinophils are refractory to most forms of transfection or viral infection. To investigate the possibility that eosinophils could take up exogenously added protein TAT-IκBα(S32A, S36A) and rIκBα were conjugated to FITC to allow their direct visualisation. TAT-IκBα(S32A, S36A)-FITC was efficiently taken up by eosinophils whereas rIκBα-FITC showed no evidence of uptake into eosinophils. To establish the ability of TAT-IκBα to inhibit NF-κB in eosinophils, TAT-IκBα(S32A, S36A) was preincubated with eosinophils prior to treatment with TNFα. Cells were fractionated into nucleus and cytoplasm and analysed by WB with IκBα and p65 antibodies respectively. As in A549 cells, TNFα induced degradation of endogenous IκBα in eosinophils, irrespective of the presence of TAT-IκBα(S32A, S36A) (figure 3B upper panel).

In the absence of TAT-IκBα(S32A, S36A) a significant amount of NF-κB p65 is detected in the nuclei of eosinophils, suggesting that a fraction of NF-κB is constitutively active in these cells. However the nuclear content of NF-κB p65 is dramatically increased

after treatment with TNFα (figure 3B, lower panel). In the presence of exogenous TAT-IκBα(S32A, S36A), the eosinophil nuclear extract is cleared of constitutively active NF-κB p65 and TNFα induced nuclear accumulation of NF-κB p65 is substantially reduced (figure 3B, lower panel).

To establish the biological consequence of inhibiting NF-κB in human eosinophils TAT-IκBα(S32A, S36A) was transduced into these cells and half of the cells treated with TNFα. After 24 hours the proportion of cells exhibiting characteristic apoptotic morphology was determined. In untreated or GST-TAT treated eosinophils 20% of cells became apoptotic after 24 hours regardless of the presence of TNFα. In the presence of TAT-IκBα(S32A, S36A), there is a dramatic increase in the proportion of apoptotic cells (78%) that is independent of TNFα treatment (figure 3C). These results suggest that the presence of constitutively active NF-κB is required for eosinophil survival.

4. Discussion

Eosinophils are notoriously difficult cells in which to carry out molecular analysis as they are refractive to transfection and viral infection. Here we establish the principle of HIV TAT Arginine rich motif peptide (YGRKKRRQRRR) mediated uptake of proteins into eosinophils and demonstrate that TAT fused to the complete IκBα protein is efficiently internalised in these cells. TAT-IκBα(S32A, S36A) efficiently enters HeLa cells and blocks signal-induced activation of NF-κB dependent transcription. In A549 cells exogenous TAT-IκBα(S32A, S36A) does not stop TNFα induced degradation of endogenous IκBα, but it does inhibit nuclear translocation of NF-κB p65. Thus it is likely that NF-κB heterodimers released from endogenous IκBα are bound by TAT-IκBα(S32A, S36A) and retained in a transcriptionally inert state in the cytoplasm. This is coincident with recently published studies employing various versions of TAT-IκBα carried out in HeLa cells, Jurkat cells and bone marrow macrophages [27, 28]. The biological consequences of introducing TAT-IκBα(S32A, S36A) into eosinophils are striking and a dramatic increase in apoptosis is observed, even in the absence of TNFα (figure 3C) although this is somewhat donor dependent. This suggests that in eosinophils constitutively active NF-κB is required to maintain a survival response and inhibition of NF-κB by TAT-IκBα(S32A, S36A) unmasks a potent pro-apoptotic signal. This is consistent with the observation that NF-κB p65 is detected in the nuclei of unstimulated eosinophils and this nuclear p65 is cleared by exogenously added TAT-IκBα(S32A, S36A) (figure 3B). Given that free IκBα has the ability to translocate to the nucleus, disengage bound NF-κB from DNA and export the NF-κB/IκBα complex back to the

cytoplasm [29], it is likely that exogenously added TAT-IκBα(S32A, S36A) inhibits NF-κB dependent transcription in eosinophils by this mechanism. A requirement for constitutive activation of NF-κB to provide a survival response was also evident from previous studies in eosinophils where pharmacological inhibitors of NF-κB activity such as gliotoxin and Mg132 induced apoptosis [5].

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Figure legends;

Figure 1; A. Schematic diagram representing the structure of TAT-IκBα(S32A, S36A). **B.** The indicated purified proteins were analysed by SDS-PAGE followed by Coomassie Blue Staining. **C.** HeLa 57A cells containing an NF-κB-dependent luciferase reporter were incubated with recombinant IκBα (rIκBα), GST-TAT or TAT-IκBα(S32A, S36A) at 30μg/ml for 30 min prior to treatment with TNFα (5ng/ml). After a further 6 hours luciferase activity was determined and expressed as fold activation relative to the activity in cells that were not exposed to TNFα. **D**. The effect of TAT-IκBα(S32A, S36A) and TAT-IκBα(WT) on NF-κB-dependent reporter activation. HeLa 57A cells were incubated with the indicated TAT-IκBα (S32A, S36A) or TAT-IκBα(WT) for 30 min at 37°C and exposed to TNFα (5ng/ml) for further 30 min. Error were not shown as they were within the range of the symbols.

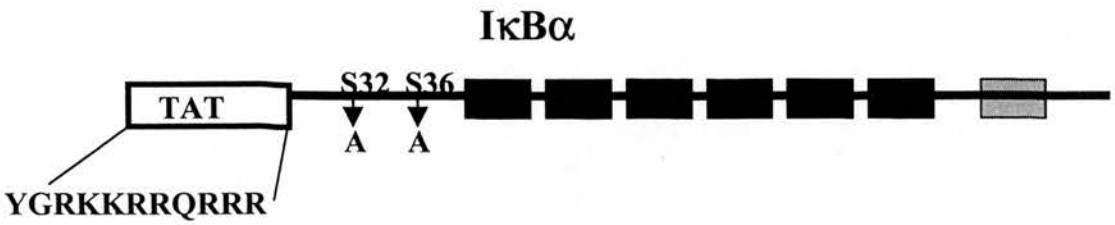
Figure 2. Inhibition of NF-κB by TAT-IκBα in A549 cells. A549 cells were incubated with TAT-IκBα(S32A, S36A) (30μg/ml) and GST-TAT (30μg/ml) for 30 min at 37°C and then treated with TNFα (5ng/ml) for 30 min. **A**. Cytoplasmic extract was analysed by WB with anti IκBα antibody. **B.** Cells were stained with anti-p65 antibody and viewed by fluorescence microscopy as indicated in materials and methods.

Figure 3. A. TAT-IκBα transduction into eosinophils FITC conjugated TAT-IκBα or rIκBα (both at 30μg/ml) were incubated with purified human eosinophils for 30 min at 37°C in the dark. After extensive washing to remove unbound material, FITC conjugated

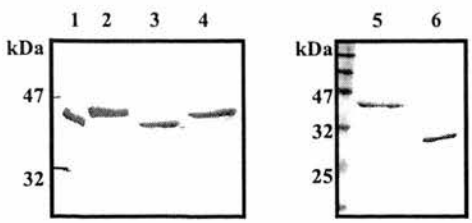
proteins were visualised by fluorescence microscopy. **B**. Eosinophils were incubated with TAT-IκBα(S32A, S36A) (30μg/ml) for 30 min at 37°C and further treated with TNFα (5ng/ml) for 30 min, prior to preparation of nuclear and cytoplasmic extracts. IκBα levels in cytoplasmic extracts and p65 levels in nuclear extracts were determined by WB. **C.** Eosinophils were treated with TAT-IκBα(S32A, S36A) (30μg/ml) and GST-TAT (30μg/ml) for 30 min at 37°C and further treated with TNFα (10ng/ml) as indicated. At 24 hours, apoptosis was determined morphologically. All values represent mean ± S. E. of n = 3 experiments. * represents significant difference from appropriate controls ($p<0.05$).

Figure 1. Fujihara et al.,

A

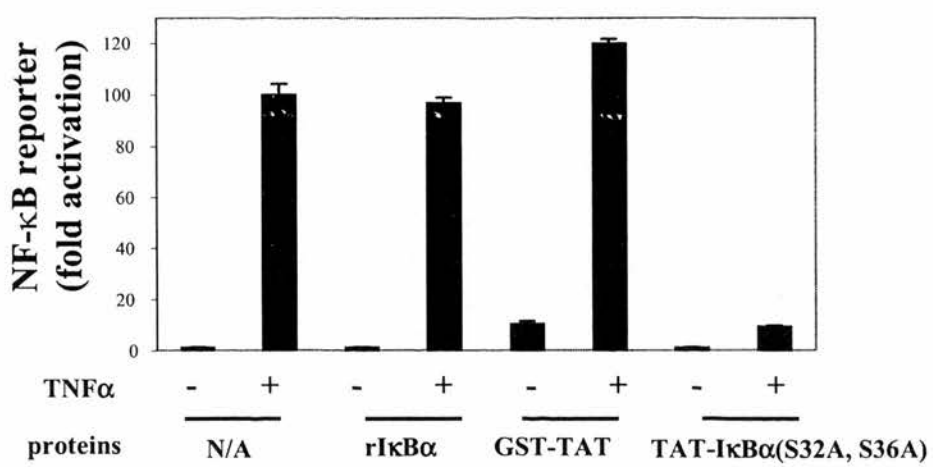


B



1. rIκBα
2. TAT-IκBα(WT)
3. rIκBα
4. TAT-IκBα(S32A, S36A)
5. rIκBα
6. GST-TAT

C



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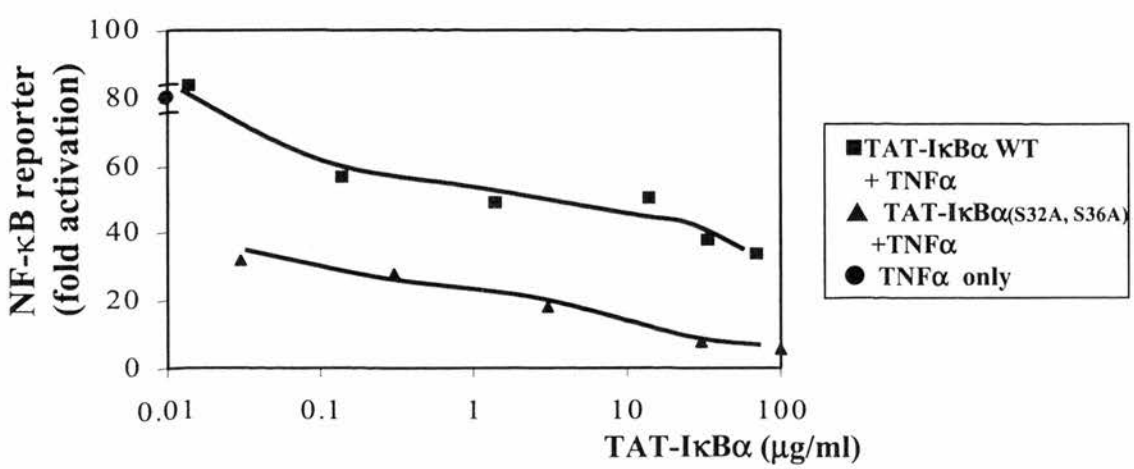

Figure 2. Fujihara et al.,

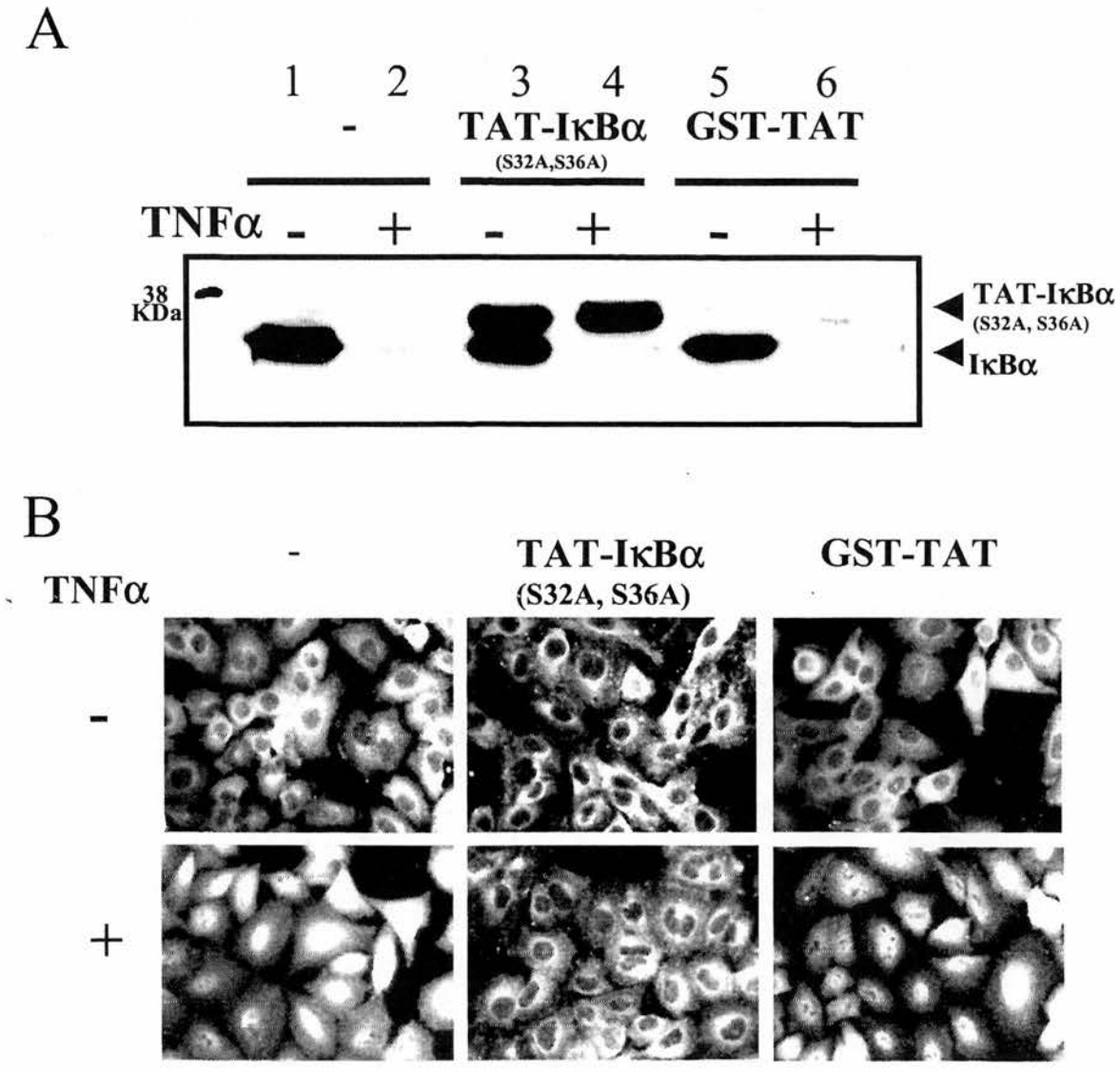

Figure 3. Fujihara et al.,